

PCT

## REQUEST

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PCT/GB 00 / 03681	
International Application No.	
25 SEPTEMBER 2000	
International Filing Date 25/09/00	
United Kingdom Patent Office PCT International Application	
Name of receiving Office and "PCT International Application"	
Applicant's or agent's file reference (if desired) (12 characters maximum) GWS/21755.01	

<b>Box No. I TITLE OF INVENTION</b>	
INHIBITION OF SECRETION FROM NON-NEURONAL CELLS	
<b>Box No. II APPLICANT</b>	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)	
FOSTER; Keith Alan MICROBIOLOGICAL RESEARCH AUTHORITY CAMR PORTON DOWN, SALISBURY SP4 0JG UNITED KINGDOM	
<input type="checkbox"/> This person is also inventor.	
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Facsimile No.	
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State (that is, country) of nationality: GB	State (that is, country) of residence: GB
This person is applicant for the purposes of: <input checked="" type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box	
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This person is:	
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<input checked="" type="checkbox"/> applicant and inventor	
<input type="checkbox"/> inventor only (If this check-box is marked, do not fill in below.)	
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<input checked="" type="checkbox"/> Further applicants and or (further) inventors are indicated on a continuation sheet.	
<b>Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE</b>	
The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: <input checked="" type="checkbox"/> agent <input type="checkbox"/> common representative	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)	
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18 DECEMBER 2000

SUBSTITUTE SHEET (RULE 25)

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☒ applicant and inventor  
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☒ applicant and inventor  
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Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

This person is:

- ☐ applicant only  
☒ applicant and inventor  
☐ inventor only (If this check-box is marked, do not fill in below.)

State (that is, country) of nationality:  
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This person is:

- ☐ applicant only  
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## Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) (mark the applicable check-boxes; at least one must be marked):

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- ☐ AP ARIPO Patent: GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, MZ Mozambique, SD Sudan, SL Sierra Leone, SZ Swaziland, TZ United Republic of Tanzania, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
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**Precautionary Designation Statement:** In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. (Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)

**Supplemental Box** If the Supplemental Box is not used, this sheet should not be included in the request.

1. If, in any of the Boxes, the space is insufficient to furnish all the information: in such case, write "Continuation of Box No. ..." (indicate the number of the Box) and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:

- (i) if more than two persons are involved as applicants and/or inventors and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;
- (ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication "the States indicated in the Supplemental Box" is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;
- (iii) if, in Box No. II or in any of the sub-boxes of Box No. III, the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;
- (iv) if, in addition to the agent(s) indicated in Box No. IV, there are further agents: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;
- (v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication "patent of addition," or "certificate of addition," or if, in Box No. V, the name of the United States of America is accompanied by an indication "continuation" or "continuation-in-part": in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;
- (vi) if, in Box No. VI, there are more than three earlier applications whose priority is claimed: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;
- (vii) if, in Box No. VI, the earlier application is an ARIPO application: in such case, write "Continuation of Box No. VI", specify the number of the item corresponding to that earlier application and indicate at least one country party to the Paris Convention for the Protection of Industrial Property or one Member of the World Trade Organization for which that earlier application was filed.

2. If, with regard to the precautionary designation statement contained in Box No. I, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.

3. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning non-prejudicial disclosures or exceptions to lack of novelty: in such case, write "Statement concerning non-prejudicial disclosures or exceptions to lack of novelty" and furnish that statement below.

Continuation of Box No. IV

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 GARRATT, Peter Douglas  
 MOIR, Michael Christopher  
 COZENS, Paul Dennis  
 SCHLICH, George William  
 COLMER, Stephen Gary  
 KAZI, Ilya  
 INGRAM, Brian Victor  
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 BRADLEY, Josephine Mary  
 MACLEAN, Martin Robert

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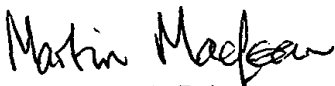
<b>Box No. VI PRIORITY CLAIM</b>		<input type="checkbox"/> Further priority claims are indicated in the Supplemental Box.		
Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is:		
		national application: country	regional application: regional Office	international application: receiving Office
item (1) 23/09/99	9922558.3	GB		
item (2)				
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) (only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office) identified above as item(s): (1)

\* Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.

<b>Box No. VII INTERNATIONAL SEARCHING AUTHORITY</b>		
Choice of International Searching Authority (ISA) (if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):	Request to use results of earlier search; reference to that search (if an earlier search has been carried out by or requested from the International Searching Authority):	
ISA / EP	Date (day/month/year)	Number Country (or regional Office)

<b>Box No. VIII CHECK LIST; LANGUAGE OF FILING</b>	
This international application contains the following number of sheets:	This international application is accompanied by the item(s) marked below:
request : 5 ✓	1. <input checked="" type="checkbox"/> fee calculation sheet
description (excluding sequence listing part) : 46 ✓	2. <input type="checkbox"/> separate signed power of attorney
claims : 7 ✓	3. <input checked="" type="checkbox"/> copy of general power of attorney; reference number, if any:
abstract : 1 ✓	4. <input type="checkbox"/> statement explaining lack of signature
drawings : 9 ✓	5. <input type="checkbox"/> priority document(s) identified in Box No. VI as item(s):
sequence listing part of description : 0	6. <input type="checkbox"/> translation of international application into (language):
Total number of sheets : 68 ✓	7. <input type="checkbox"/> separate indications concerning deposited microorganism or other biological material
	8. <input type="checkbox"/> nucleotide and/or amino acid sequence listing in computer readable form
	9. <input checked="" type="checkbox"/> other (specify): PF 23/77
Figure of the drawings which should accompany the abstract: 3	Language of filing of the international application: ENGLISH

<b>Box No. IX SIGNATURE OF APPLICANT OR AGENT</b>	
Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).	
 MACLEAN, Martin Robert	

For receiving Office use only		2. Drawings: <input checked="" type="checkbox"/> received: <input type="checkbox"/> not received:
1. Date of actual receipt of the purported international application:	25 SEPTEMBER 2000 25/09/00	
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:		
4. Date of timely receipt of the required corrections under PCT Article 11(2):		
5. International Searching Authority (if two or more are competent): ISA /	6. <input type="checkbox"/> Transmittal of search copy delayed until search fee is paid.	

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## INHIBITION OF SECRETION FROM NON-NEURONAL CELLS

5 The present invention relates to treatment of disease by inhibition of cellular secretory processes, to agents and compositions therefor, and to manufacture of those agents and compositions. The present invention relates particularly, to treatment of diseases dependent upon the exocytotic activity of endocrine cells, exocrine cells, inflammatory cells, cells of the immune system, cells of the cardiovascular system and bone cells.

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Exocytosis is the fusion of secretory vesicles with the plasma membrane and results in the discharge of vesicle content - a process also known as cell secretion. Exocytosis can be constitutive or regulated. Constitutive exocytosis is thought to occur in every cell type whereas regulated  
15 exocytosis occurs from specialised cells.

The understanding of the mechanisms involved in exocytosis has increased rapidly, following the proposal of the SNARE hypothesis (Rothman, 1994, Nature 372, 55-63). This hypothesis describes protein markers on vesicles, which recognise target membrane markers. These so-called cognate  
20 SNARES (denoted v-SNARE for vesicle and t-SNARE for target) facilitate docking and fusion of vesicles with the correct membranes, thus directing discharge of the vesicular contents into the appropriate compartment. Key to the understanding of this process has been the identification of the  
25 proteins involved. Three SNARE protein families have been identified for exocytosis: SNAP-25 and SNAP-23, and syntaxins are the t-SNARE families in the membrane; and VAMPs (vesicle-associated membrane protein), including synaptobrevin and cellubrevin, are the v-SNARE family on secretory vesicles. Key components of the fusion machinery including  
30 SNARES are involved in both regulated and constitutive exocytosis (De Camilli, 1993, Nature, 364, 387-388).

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The clostridial neurotoxins are proteins with molecular masses of the order of 150kDa. They are produced by various species of the genus *Clostridium*, most importantly *C. tetani* and several strains of *C. botulinum*. There are at present eight different classes of the neurotoxins known: tetanus toxin and botulinum neurotoxin in its serotypes A, B, C<sub>1</sub>, D, E, F and G, and they all share similar structures and modes of action. The clostridial neurotoxins are synthesized by the bacterium as a single polypeptide that is modified post-translationally to form two polypeptide chains joined together by a disulphide bond. The two chains are termed the heavy chain (H) which has a molecular mass of approximately 100 kDa and the light chain (LC) which has a molecular mass of approximately 50 kDa. The clostridial neurotoxins are highly selective for neuronal cells, and bind with high affinity thereto [see Black, J.D. and Dolly, J.O. (1987) *Selective location of acceptors for BoNT/A in the central and peripheral nervous systems. Neuroscience*, 23, pp.767-779; Habermann, E. and Dreyer, F. (1986) *Clostridial neurotoxins: handling and action at the cellular and molecular level. Curr. Top. Microbiol. Immunol.* 129, pp.93-179; and Sugiyama, H. (1980) *Clostridium botulinum neurotoxin. Microbiol. Rev.*, 44, pp.419-448 (and internally cited references)].

The functional requirements of neurointoxication by the clostridial neurotoxins can be assigned to specific domains within the neurotoxin structure. The clostridial neurotoxins bind to an acceptor site on the cell membrane of the motor neuron at the neuromuscular junction and, following binding to the highly specific receptor, are internalised by an endocytotic mechanism. The specific neuromuscular junction binding activity of clostridial neurotoxins is known to reside in the carboxy-terminal portion of the heavy chain component of the dichain neurotoxin molecule, a region known as H<sub>C</sub>. The internalised clostridial neurotoxins possess a highly specific zinc-dependent endopeptidase activity that hydrolyses a specific peptide bond in at least one of three protein families, synaptobrevin, syntaxin or SNAP-25, which are crucial components of the

- 3 -

neurosecretory machinery. The zinc-dependent endopeptidase activity of clostridial neurotoxins is found to reside in the L-chain (LC). The amino-terminal portion of the heavy chain component of the dichain neurotoxin molecule, a region known as  $H_N$ , is responsible for translocation of the neurotoxin, or a portion of it containing the endopeptidase activity, across the endosomal membrane following internalisation, thus allowing access of the endopeptidase to the neuronal cytosol and its substrate protein(s). The result of neurointoxication is inhibition of neurotransmitter release from the target neuron due to prevention of release of synaptic vesicle contents.

The mechanism by which the  $H_N$  domain effects translocation of the endopeptidase into the neuronal cytosol is not fully characterised but is believed to involve a conformational change, insertion into the endosomal membrane and formation of some form of channel or pore through which the endopeptidase can gain access to the neuronal cytosol. Following binding to its specific receptor at the neuronal surface pharmacological and morphologic evidence indicate that the clostridial neurotoxins enter the cell by endocytosis [Black & Dolly (1986) J. Cell Biol. 103, 535-44] and then have to pass through a low pH step for neuron intoxication to occur [Simpson et al (1994) J. Pharmacol Exp. Ther., 269, 256-62]. Acidic pH does not activate the toxin directly via a structural change, but is believed to trigger the process of LC membrane translocation from the neuronal endosomal vesicle lumen to the neuronal cytosol [Montecucco et al (1994) FEBS Lett. 346, 92-98]. There is a general consensus that toxin-determined channels are related to the translocation process into the cytosol [Schiavo & Montecucco (1997) in Bacterial Toxins (ed. K. Aktories)]. This model requires that the  $H_N$  domain forms a transmembrane hydrophobic pore across the acidic vesicle membrane that allows the partially unfolded LC passage through to the cytosol. The requisite conformational change is believed to be triggered by environmental factors in the neuronal endosomal compartment into which the neurotoxin is internalised, and a necessary feature of the binding domain of the  $H_C$  is to

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target binding sites which enable internalisation into the appropriate endosomal compartment. Therefore clostridial neurotoxins have evolved to target cell surface moieties that fulfil this requirement.

5       Hormones are chemical messengers that are secreted by the endocrine glands of the body. They exercise specific physiological actions on other organs to which they are carried by the blood. The range of processes regulated by hormones includes various aspects of homeostasis (e.g. insulin regulates the concentration of glucose in the blood), growth (e.g. growth hormone promotes growth and regulates fat, carbohydrate and protein metabolism), and maturation (e.g. sex hormones promote sexual maturation and reproduction). Endocrine hyperfunction results in disease conditions which are caused by excessive amounts of a hormone or hormones in the bloodstream. The causes of hyperfunction are classified as neoplastic, autoimmune, iatrogenic and inflammatory. The endocrine hyperfunction disorders are a complex group of diseases, not only because there is a large number of glands that can cause a pathology (e.g. anterior pituitary, posterior pituitary, thyroid, parathyroid, adrenal cortex, adrenal medulla, pancreas, ovaries, testis) but because many of the glands produce more than one hormone (e.g. the anterior pituitary produces corticotrophin, prolactin, luteinizing hormone, follicle stimulating hormone, thyroid stimulating hormone and gonadotrophins). The majority of disorders that cause hormone excess are due to neoplastic growth of hormone producing cells. However, certain tumours of non-endocrine origin can synthesise hormones causing endocrine hyperfunction disease symptoms. The hormone production under these conditions is termed "ectopic". Surgical removal or radiation induced destruction of part or all of the hypersecreting tissue is frequently the treatment of choice. However, these approaches are not always applicable, result in complete loss of hormone production or have to be repeated due to re-growth of the secreting tissue.

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A further level of complexity in endocrine hyperfunction disorders arises in

- 5 -

a group of conditions termed multiple endocrine neoplasia (MEN) where two or more endocrine glands are involved. The multiple endocrine neoplasia syndromes (MEN1 and MEN2) are familial conditions with an autosomal dominant pattern of inheritance. MEN1 is characterised by the association of parathyroid hyperplasia, pancreatic endocrine tumours, and pituitary adenomas, and has a prevalence of about 1 in 10000. MEN2 is the association of medullary cell carcinoma of the thyroid and phaeochromocytoma, though parathyroid hyperplasia may also occur in some sufferers.

Most of the morbidity associated with MEN1 is due to the effects of pancreatic endocrine tumours. Often surgery is not possible and the therapeutic aim is to reduce hormone excess. Aside from reducing tumour bulk, which is often precluded, inhibition of hormone secretion is the preferred course of action. Current procedures include subcutaneous application of the somatostatin analogue, octreotide. However, this approach is only temporarily effective, and the success diminishes over a period of months.

Many further disease states are known that involve secretion from other non-endocrine, non-neuronal cells. It would accordingly be desirable to treat, reduce or prevent secretion by non-neuronal cells, such as hyperfunction of the endocrine cells that causes or leads to these disease conditions.

The activity of the botulinum neurotoxins is exclusively restricted to inhibition of neurotransmitter release from neurons. This is due to the exclusive expression of high affinity binding sites for clostridial neurotoxins on neuronal cells [see Daniels-Holgate, P.U. and Dolly, J.O. (1996) *Productive and non-productive binding of botulinum neurotoxin to motor nerve endings are distinguished by its heavy chain. J. Neurosci. Res.* 44, 263-271].

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Non-neuronal cells do not possess the high affinity binding sites for clostridial neurotoxins, and are therefore refractory to the inhibitory effects of exogenously applied neurotoxin. Simple application of clostridial neurotoxins to the surface of non-neuronal cells does not therefore lead to inhibition of secretory vesicle exocytosis.

The productive binding or lack of productive binding of clostridial neurotoxins thereby defines neuronal and non-neuronal cells respectively.

In addition to lacking high affinity binding sites for clostridial neurotoxins, absence of the correct internalisation and intracellular routing mechanism, or additional factors that are not yet understood, would prevent clostridial neurotoxin action in non-neuronal cells.

It is known from WO96/33273 that hybrid clostridial neurotoxins endopeptidases can be prepared and that these hybrids effectively inhibit release of neurotransmitters from neuronal cells to which they are targeted, such as pain transmitting neurons. WO96/33273 describes the activity of hybrids only in neuronal systems where neuronal mechanisms of internalisation and vesicular routing are operational.

Non-neuronal cells are, however, refractory to the effects of clostridial neurotoxins, since simple application of clostridial neurotoxins to the surface of non-neuronal cells does not lead to inhibition of secretory vesicle exocytosis. This insensitivity of non-neuronal cells to clostridial neurotoxins may be due to absence of the requisite receptor, absence of the correct internalisation & intracellular routing mechanism, or additional factors that are not yet understood.

WO95/17904 describes the use of *C. botulinum* holotoxin in the treatment of various disorders such as excessive sweating, lacrimation and mucus secretion, and pain. WO95/17904 describes treatment by targeting

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neuronal cells

It is an object of the present invention to provide methods and compositions for inhibition of secretion from non-neuronal cells.

5

Accordingly, the present invention is based upon the use of a composition which inhibits the exocytotic machinery in neuronal cells and which surprisingly has been found to be effective at inhibiting exocytotic processes in non-neuronal cells.

10

A first aspect of the invention thus provides a method of inhibiting secretion from a non-neuronal cell comprising administering an agent comprising at least first and second domains, wherein the first domain cleaves one or more proteins essential to exocytosis and the second domain translocates the first domain into the cell.

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Advantageously, the invention provides for inhibition of non-neuronal secretion and enables treatment of disease caused, exacerbated or maintained by such secretion.

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An agent for use in the invention is suitably prepared by replacement of the cell-binding H<sub>C</sub> domain of a clostridial neurotoxin with a ligand capable of binding to the surface of non-neuronal cells. Surprisingly, this agent is capable of inhibiting the exocytosis of a variety of secreted substances from non-neuronal cells. By covalently linking a clostridial neurotoxin, or a hybrid of two clostridial neurotoxins, in which the H<sub>C</sub> region of the H-chain has been removed or modified, to a new molecule or moiety, the Targeting Moiety (TM), an agent is produced that binds to a binding site (BS) on the surface of the relevant non-neuronal secretory cells. A further surprising aspect of the present invention is that if the L-chain of a clostridial neurotoxin, or a fragment, variant or derivative of the L-chain containing the endopeptidase activity, is covalently linked to a TM which

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can also effect internalisation of the L-chain, or a fragment of the endopeptidase activity, into the cytoplasm of a non-neuronal secretory cell, this also produces an agent capable of inhibiting secretion. Thus, the present invention overcomes the insusceptibility of non-neuronal cells to the inhibitory effects of clostridial neurotoxins.

An example of an agent of the invention is a polypeptide comprising first and second domains, wherein said first domain cleaves one or more vesicle or plasma-membrane associated proteins essential to neuronal exocytosis and wherein said second domain translocates the polypeptide into the cell or translocates at least that portion responsible for the inhibition of exocytosis into the non-neuronal cell. The polypeptide can be derived from a neurotoxin in which case the polypeptide is typically free of clostridial neurotoxin and free of any clostridial neurotoxin precursor that can be converted into toxin by proteolytic action, being accordingly substantially non-toxic and suitable for therapeutic use. Accordingly, the invention may thus use polypeptides containing a domain equivalent to a clostridial toxin light chain and a domain providing the translocation function of the H<sub>N</sub> of a clostridial toxin heavy chain, whilst lacking the functional aspects of a clostridial toxin H<sub>C</sub> domain.

In use of the invention, the polypeptide is administered *in vivo* to a patient, the first domain is translocated into a non-neuronal cell by action of the second domain and cleaves one or more vesicle or plasma-membrane associated proteins essential to the specific cellular process of exocytosis, and cleavage of these proteins results in inhibition of exocytosis, thereby resulting in inhibition of secretion, typically in a non-cytotoxic manner.

The polypeptide of the invention may be obtained by expression of a recombinant nucleic acid, preferably a DNA, and can be a single polypeptide, that is to say not cleaved into separate light and heavy chain domains or two polypeptides linked for example by a disulphide bond.

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The first domain preferably comprises a clostridial toxin light chain or a functional fragment or variant of a clostridial toxin light chain. The fragment is optionally an N-terminal, or C-terminal fragment of the light chain, or is an internal fragment, so long as it substantially retains the ability to cleave the vesicle or plasma-membrane associated protein essential to exocytosis. The minimal domains necessary for the activity of the light chain of clostridial toxins are described in J. Biol. Chem., Vol.267, No. 21, July 1992, pages 14721-14729. The variant has a different peptide sequence from the light chain or from the fragment, though it too is capable of cleaving the vesicle or plasma-membrane associated protein. It is conveniently obtained by insertion, deletion and/or substitution of a light chain or fragment thereof. A variety of variants are possible, including (i) an N-terminal extension to a clostridial toxin light chain or fragment (ii) a clostridial toxin light chain or fragment modified by alteration of at least one amino acid (iii) a C-terminal extension to a clostridial toxin light chain or fragment, or (iv) combinations of 2 or more of (i)-(iii). In further embodiments of the invention, the variant contains an amino acid sequence modified so that (a) there is no protease sensitive region between the LC and H<sub>N</sub> components of the polypeptide, or (b) the protease sensitive region is specific for a particular protease. This latter embodiment is of use if it is desired to activate the endopeptidase activity of the light chain in a particular environment or cell, though, in general, the polypeptides of the invention are in an active form prior to administration.

The first domain preferably exhibits endopeptidase activity specific for a substrate selected from one or more of SNAP-25, synaptobrevin/VAMP and syntaxin. The clostridial toxin from which this domain can be obtained or derived is preferably botulinum toxin or tetanus toxin. The polypeptide can further comprise a light chain or fragment or variant of one toxin type and a heavy chain or fragment or variant of another toxin type.

The second domain preferably comprises a clostridial toxin heavy chain H<sub>N</sub>

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portion or a fragment or variant of a clostridial toxin heavy chain  $H_N$  portion. The fragment is optionally an N-terminal or C-terminal or internal fragment, so long as it retains the function of the  $H_N$  domain. Teachings of regions within the  $H_N$  responsible for its function are provided for example in Biochemistry 1995, 34, pages 15175-15181 and Eur. J. Biochem, 1989, 185, pages 197-203. The variant has a different sequence from the  $H_N$  domain or fragment, though it too retains the function of the  $H_N$  domain. It is conveniently obtained by insertion, deletion and/or substitution of a  $H_N$  domain or fragment thereof, and examples of variants include (i) an N-terminal extension to a  $H_N$  domain or fragment, (ii) a C-terminal extension to a  $H_N$  domain or fragment, (iii) a modification to a  $H_N$  domain or fragment by alteration of at least one amino acid, or (iv) combinations of 2 or more of (i)-(iii). The clostridial toxin is preferably botulinum toxin or tetanus toxin.

In preparation of the polypeptides by recombinant means, methods employing fusion proteins can be employed, for example a fusion protein comprising a fusion of (a) a polypeptide of the invention as described above with (b) a second polypeptide adapted for binding to a chromatography matrix so as to enable purification of the fusion protein using said chromatography matrix. It is convenient for the second polypeptide to be adapted to bind to an affinity matrix, such as glutathione Sepharose, enabling rapid separation and purification of the fusion protein from an impure source, such as a cell extract or supernatant.

One second purification polypeptide is glutathione-S-transferase (GST), and others may be chosen so as to enable purification on a chromatography column according to conventional techniques.

In a second aspect of the invention there is provided a method of inhibiting secretion from selected non-neuronal cells responsible for regulated secretion by administering an agent of the invention.

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In a third aspect of the invention there is provided a method of treatment of disease resulting, or caused or maintained by secretions from non-neuronal cells, comprising administering an agent of the invention.

5 In further aspects of the invention there are provided agents of the invention targeted to non-neuronal cells responsible for secretion.

In one embodiment of the invention, an agent is provided for the treatment of conditions resulting from hyperfunction of endocrine cells, for example  
10 endocrine neoplasia.

Accordingly, an agent of the invention is used in the treatment of endocrine hyperfunction, to inhibit secretion of endocrine cell-derived chemical messengers. An advantage of the invention is that effective treatment of  
15 endocrine hyperfunction and associated disease states is now provided, offering relief to sufferers where hitherto there was none and no such agent available.

A further advantage of the invention is that agents are made available  
20 which, in use, result in the inhibition of or decrease in hypersecretion of multiple hormones from a single endocrine gland. Thus, the multitude of disorders that result from hyperfunction of one gland (eg. the anterior pituitary) will be simultaneously treated by a reduction in the function of the hypersecreting gland.

25 The agent preferably comprises a ligand or targeting domain which binds to an endocrine cell, and is thus rendered specific for these cell types. Examples of suitable ligands include iodine; thyroid stimulating hormone (TSH); TSH receptor antibodies; antibodies to the islet-specific monosialoganglioside GM2-1; insulin, insulin-like growth factor and antibodies to the  
30 receptors of both; TSH releasing hormone (protirelin) and antibodies to its receptor; FSH/LH releasing hormone (gonadorelin) and antibodies to its

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receptor; corticotrophin releasing hormone (CRH) and antibodies to its receptor; and ACTH and antibodies to its receptor. According to the invention, an endocrine targeted agent may thus be suitable for the treatment of a disease selected from: endocrine neoplasia including MEN;  
5 thyrotoxicosis and other diseases dependent on hypersecretions from the thyroid; acromegaly, hyperprolactinaemia, Cushings disease and other diseases dependent on anterior pituitary hypersecretion; hyperandrogenism, chronic anovulation and other diseases associated with polycystic ovarian syndrome.

10 In a further embodiment, an agent of the invention is used for the treatment of conditions resulting from secretions of inflammatory cells, for example allergies. Ligands suitable to target agent to these cells include  
15 (i) for mast cells, complement receptors in general, including C4 domain of the Fc IgE, and antibodies/ligands to the C3a/C4a-R complement receptor; (ii) for eosinophils, antibodies/ligands to the C3a/C4a-R complement receptor, anti VLA-4 monoclonal antibody, anti-IL5 receptor, antigens or antibodies reactive toward CR4 complement receptor; (iii) for macrophages and monocytes, macrophage stimulating factor, (iv) for macrophages,  
20 monocytes and neutrophils, bacterial LPS and yeast B-glucans which bind to CR3, (v) for neutrophils, antibody to OX42, an antigen associated with the iC3b complement receptor, or IL8; (vi) for fibroblasts, mannose 6-phosphate/insulin-like growth factor-beta (M6P/IGF-II) receptor and PA2.26, antibody to a cell-surface receptor for active fibroblasts in mice. Diseases  
25 thus treatable according to the invention include diseases selected from allergies (seasonal allergic rhinitis (hay fever), allergic conjunctivitis, vasomotor rhinitis and food allergy), eosinophilia, asthma, rheumatoid arthritis, systemic lupus erythematosus, discoid lupus erythematosus, ulcerative colitis, Crohn's disease, haemorrhoids, pruritus,  
30 glomerulonephritis, hepatitis, pancreatitis, gastritis, vasculitis, myocarditis, psoriasis, eczema, chronic radiation-induced fibrosis, lung scarring and other fibrotic disorders.

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VAMP expression has been demonstrated in B-lymphocytes [see Olken, S. K. and Corley, R. B. 1998, *Mol. Biol. Cell.* 9, 207a]. Thus, an agent according to the present invention, when targeted to a B-lymphocyte and following internalisation and retrograde transport, may exert its inhibitory effect on such target cells.

In a further embodiment, an agent of the invention is provided for the treatment of conditions resulting from secretions of the exocrine cells, for example acute pancreatitis (Hansen *et al.*, 1999, *J. Biol. Chem.* 274, 22871-22876). Ligands suitable to target agent to these cells include pituitary adenylyl cyclase activating peptide (PACAP-38) or an antibody to its receptor. The present invention also concerns treatment of mucus hypersecretion from mucus-secreting cells located in the alimentary tract, in particular located in the colon.

Gaisano, H. Y. *et al.* (1994) *J. Biol. Chem.* 269, pp.17062-17066 has demonstrated that, following *in vitro* membrane permeabilisation to permit cellular entry, tetanus toxin light chain cleaves a vesicle-associated membrane protein (VAMP) isoform 2 in rat pancreatic zymogen granules, and inhibits enzyme secretion. Thus, an agent according to the present invention, when targeted to a pancreatic cell and following internalisation and retrograde transport, may exert its inhibitory effect on such target cells.

In a further embodiment, an agent of the invention is used for the treatment of conditions resulting from secretions of immunological cells, for example autoimmune disorders where B lymphocytes are to be targeted (immunosuppression). Ligands suitable to target agent to these cells include Epstein Barr virus fragment/surface feature or idiotypic antibody (binds to CR2 receptor on B-lymphocytes and lymph node follicular dendritic cells). Diseases treatable include myasthenia gravis, rheumatoid arthritis, systemic lupus erythematosus, discoid lupus erythematosus,

organ transplant, tissue transplant, fluid transplant, Graves disease, thyrotoxicosis, autoimmune diabetes, haemolytic anaemia, thrombocytopenic purpura, neutropenia, chronic autoimmune hepatitis, autoimmune gastritis, pernicious anaemia, Hashimoto's thyroiditis, Addison's disease, Sjogren's syndrome, primary biliary cirrhosis, polymyositis, scleroderma, systemic sclerosis, pemphigus vulgaris, bullous pemphigoid, myocarditis, rheumatic carditis, glomerulonephritis (Goodpasture type), uveitis, orchitis, ulcerative colitis, vasculitis, atrophic gastritis, pernicious anaemia, type 1 diabetes mellitus.

By using cell permeabilisation techniques it has been possible to internalise BoNT/C into eosinophils [see Pinxteren JA, *et al* (2000) *Biochimie, Apr;82(4):385-93 Thirty years of stimulus-secretion coupling: from  $Ca^{2+}$  to GTP in the regulation of exocytosis*]. Following internalisation, BoNT/C exerted an inhibitory effect on exocytosis in eosinophils. Thus, an agent according to the present invention, when targeted to an eosinophil and following internalisation and retrograde transport, may exert its inhibitory effect on such target cells.

In a further embodiment of the invention, an agent is provided for the treatment of conditions resulting from secretions of cells of the cardiovascular system. Suitable ligands for targeting platelets for the treatment of disease states involving inappropriate platelet activation and thrombus formation include thrombin and TRAP (thrombin receptor agonist peptide) or antibodies to CD31/PECAM-1, CD24 or CD106/VCAM-1, and ligands for targeting cardiovascular endothelial cells for the treatment of hypertension include GP1b surface antigen recognising antibodies.

In a further embodiment of the invention, an agent is provided for the treatment of bone disorders. Suitable ligands for targeting osteoblasts for the treatment of a disease selected from osteopetrosis and osteoporosis include calcitonin, and for targeting an agent to osteoclasts include

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osteoclast differentiation factors (eg. TRANCE, or RANKL or OPGL), and an antibody to the receptor RANK.

5 A further specific embodiment of the present invention lies in treating mucus hypersecretion by administering a composition that inhibits mucus secretion by mucus secreting cells and/or inhibits neurotransmitter release from neuronal cells that control or direct mucus secretion. Specific disease states caused by or exacerbated by hypersecretion are localised to the airways, and are treatable by topical administration to the airways or to a  
10 selected region or to a selected portion of the airways of a compound that inhibits exocytosis in mucus secreting cells or in neurones that control or direct mucus secretion.

15 In use of the invention, a Targeting moiety (TM) provides specificity for the BS on the relevant non-neuronal secretory cells. The TM component of the agent can comprise one of many cell binding molecules, including, but not limited to, antibodies, monoclonal antibodies, antibody fragments (Fab, F(ab)'<sub>2</sub>, Fv, ScFv, etc.), lectins, hormones, cytokines, growth factors, peptides, carbohydrates, lipids, glycons, nucleic acids or complement  
20 components.

The TM is selected in accordance with the desired cell-type to which the agent of the present invention is to be targeted, and preferably has a high specificity and/or affinity for non-neuronal target cells. Preferably, the TM  
25 does not substantially bind to neuronal cells of the presynaptic muscular junction, and thus the agent is substantially non-toxic in that it is not capable of effecting muscular paralysis. This is in contrast to clostridial holotoxin which targets the presynaptic muscular junction and effects muscular paralysis. In addition, preferably the TM does not substantially  
30 bind to neuronal peripheral sensory cells, and thus the agent does not exert any substantial analgesic effect. Preferably, the TM does not substantially bind to neuronal cells, and does not therefore permit the agent to exert an

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inhibitory effect on secretion in a neuronal cell.

It is known in the art that the H<sub>C</sub> portion of the neurotoxin molecule can be removed from the other portion of the H-chain, known as H<sub>N</sub>, such that the H<sub>N</sub> fragment remains disulphide linked to the L-chain of the neurotoxin providing a fragment known as LH<sub>N</sub>. Thus, in one embodiment of the present invention the LH<sub>N</sub> fragment of a clostridial neurotoxin is covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention, the H<sub>C</sub> domain of a clostridial neurotoxin is mutated, blocked or modified, e.g. by chemical modification, to reduce or preferably incapacitate its ability to bind the neurotoxin to receptors at the neuromuscular junction. This modified clostridial neurotoxin is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention, the heavy chain of a clostridial neurotoxin, in which the H<sub>C</sub> domain is mutated, blocked or modified, e.g. by chemical modification, to reduce or preferably incapacitate its ability to bind the neurotoxin to receptors at the neuromuscular junction, is combined with the L-chain of a different clostridial neurotoxin. This hybrid, modified clostridial neurotoxin is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention, the H<sub>N</sub> domain of a clostridial neurotoxin is combined with the L-chain of a different clostridial neurotoxin. This hybrid LH<sub>N</sub> is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention, the light chain of a clostridial neurotoxin, or a fragment of the light chain containing the endopeptidase

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activity, is covalently linked, using linkages which may include one or more spacer regions, to a TM which can also effect the internalisation of the L-chain, or a fragment of the L-chain containing the endopeptidase activity, into the cytoplasm of the relevant non-neuronal cells responsible for secretion.

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In another embodiment of the invention, the light chain of a clostridial neurotoxin, or a fragment of the light chain containing the endopeptidase activity, is covalently linked, using linkages which may include one or more spacer regions, to a translocation domain to effect transport of the endopeptidase fragment into the cytosol. Examples of translocation domains derived from bacterial neurotoxins are as follows:

10

Botulinum type A neurotoxin – amino acid residues (449-871)

Botulinum type B neurotoxin – amino acid residues (441-858)

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Botulinum type C neurotoxin – amino acid residues (442-866)

Botulinum type D neurotoxin – amino acid residues (446-862)

Botulinum type E neurotoxin – amino acid residues (423-845)

Botulinum type F neurotoxin – amino acid residues (440-864)

Botulinum type G neurotoxin – amino acid residues (442-863)

20

Tetanus neurotoxin – amino acid residues (458-879)

other clostridial sources include - *C. butyricum*, and *C. argentinense*

[for the genetic basis of toxin production in *Clostridium botulinum* and *C. tetani*, see Henderson *et al* (1997) in *The Clostridia: Molecular Biology and Pathogenesis*, Academic press].

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In addition to the above translocation domains derived from clostridial sources, other non-clostridial sources may be employed in an agent according to the present invention. These include, for example, diphtheria toxin [London, E. (1992) *Biochem. Biophys. Acta.*, 1112, pp.25-51], Pseudomonas exotoxin A [Prior *et al* (1992) *Biochem.*, 31, pp.3555-3559], influenza virus haemagglutinin fusogenic peptides [Wagner *et al* (1992) *PNAS*, 89, pp.7934-7938], and amphiphilic peptides [Murata *et al* (1992)

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*Biochem.*, 31, pp.1986-1992].

In use, the domains of an agent according to the present invention are associated with each other. In one embodiment, two or more of the Domains may be joined together either directly (eg. by a covalent linkage), or via a linker molecule. Conjugation techniques suitable for use in the present invention have been well documented:-

Chemistry of protein conjugation and cross-linking. Edited by Wong, S. S. 1993, CRC Press Inc., Florida; and

Bioconjugate techniques, Edited by Hermanson, G. T. 1996, Academic Press, London, UK.

Direct linkage of two or more of Domains is now described with reference to clostridial neurotoxins and to the present Applicant's nomenclature of clostridial neurotoxin domains, namely Domain B (contains the binding domain), Domain T (contains the translocation domain) and Domain E (contains the protease domain), although no limitation thereto is intended.

In one embodiment of the present invention, Domains E and T may be mixed together in equimolar quantities under reducing conditions and covalently coupled by repeated dialysis (eg. at 4° C, with agitation), into physiological salt solution in the absence of reducing agents. At this stage, in contrast to Example 6 of WO94/21300, the E-T complex is not blocked by iodoacetamide, therefore any remaining free -SH groups are retained.

Domain B is then modified, for example, by derivatisation with SPDP followed by subsequent reduction. In this reaction, SPDP does not remain attached as a spacer molecule to Domain B, but simply increases the efficiency of this reduction reaction.

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Reduced domain B and the E-T complex may then be mixed under non-reducing conditions (eg. at 4 °C) to form a disulphid -linked E-T-B "agent".

5 In another embodiment, a coupled E-T complex may be prepared according to Example 6 of WO94/21300, including the addition of iodoacetamide to block free sulphydryl groups. However, the E-T complex is not further derivatised, and the remaining chemistry makes use of the free amino (-NH<sub>2</sub>) groups on amino acid side chains (eg. lysine, and arginine amino acids).

10 Domain B may be derivatised using carbodiimide chemistry (eg. using EDC) to activate carboxyl groups on amino acid side chains (eg. glutamate, and aspartate amino acids), and the E-T complex mixed with the derivatised Domain B to result in a covalently coupled (amide bond) E-T-B complex.

15 Suitable methodology for the creation of such an agent is, for example, as follows:-

20 Domain B was dialysed into MES buffer (0.1 M MES, 0.1 M sodium chloride, pH 5.0) to a final concentration of 0.5mg/ml. EDAC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) was added to final concentrations of 0.2 mg/ml and reacted for 30 min at room temperature. Excess EDAC was removed by desalting over a MES buffer equilibrated PD-10 column (Pharmacia). The derivatised domain B was concentrated (to  
25 >2mg/ml) using Millipore Biomax 10 concentrators. The E-T complex (1 mg/ml) was mixed for 16 hours at 4 °C, and the E-T-B complex purified by size-exclusion chromatography over a Superose 12 HR10/30 column (Pharmacia) to remove unreacted Domain B (column buffer: 50mM sodium phosphate pH6.5 + 20mM NaCl).

30 As an alternative to direct covalent linkage of the various Domains of an agent according to the present invention, suitable spacer molecules may be

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employed. The term linker molecule is used synonymously with spacer molecule. Spacer technology was readily available prior to the present application.

5 For example, one particular coupling agent (SPDP) is described in Example 6 of WO94/21300 (see lines 3-5 on page 16). In Example 6, SPDP is linked to an E-T complex, thereby providing an E-T complex including a linker molecule. This complex is then reacted a Domain B, which becomes attached to the E-T complex via the linker molecule. In this method, SPDP  
10 results in a spacing region of approximately 6.8 Angstroms between different Domains of the "agent" of the present invention.

A variant of SPDP known as LC-SPDP is identical in all respects to SPDP but for an increased chain length. LC-SPDP may be used to covalently link  
15 two Domains of the "agent" of the present invention resulting in a 15.6 Angstrom spacing between these Domains.

Examples of spacer molecules include, but are not limited to:-

20	(GGGGS) <sub>2</sub> , elbow regions of Fab	- [see Anand <i>et al.</i> (1991) J. Biol. Chem. <b>266</b> , 21874-9];
	(GGGGS) <sub>3</sub>	- [see Brinkmann <i>et al.</i> (1991) Proc. Natl. Acad. Sci. <b>88</b> , 8616-20];
25	the interdomain linker of cellulase	- [see Takkinen <i>et al.</i> (1991) Protein Eng, <b>4</b> , 837-841];
	PPPIEGR	- [see Kim (1993) Protein Science, <b>2</b> , 348-356];
	Collagen-like spacer	- [see Rock (1992) Protein Engineering, vol 5, No 6, pp583-591]; and
30	Trypsin-sensitive diphtheria	

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toxin peptide

- [see O'Hare (1990) FEBS, vol  
273, No 1,2, pp 200-204].

5 In a further embodiment of the present invention, an agent having the  
structure E-X-T-X-B, where "X" is a spacer molecule between each domain,  
may be prepared, for example, as follows:-

10 Domain E is derivatised with SPDP, but not subsequently reduced. This  
results in an SPDP-derivatised Domain E.

Domain T is similarly prepared, but subsequently reduced with 10mM  
dithiothreitol (DTT). The 10mM DTT present in the Domain T preparation,  
following elution from the QAE column (see Example 6 in WO94/21300),  
is removed by passage of Domain T through a sephadex G-25 column  
15 equilibrated in PBS.

Domain T free of reducing agent is then mixed with the SPDP-derivatised  
Domain E, with agitation at 4 °C for 16 hours. E-T complex is isolated  
from free Domain E and from free Domain T by size-exclusion  
20 chromatography (Sephadex G-150). Whereafter, the same procedure can  
be followed as described in Example 6 of WO94/21300 for rederivatisation  
of the E-T complex with SPDP, and subsequent coupling thereof to the free  
sulphydryl on Domain B.

25 The agents according to the present invention may be prepared  
recombinantly.

In one embodiment, the preparation of a recombinant agent may involve  
arrangement of the coding sequences of the selected TM and clostridial  
30 neurotoxin component in a single genetic construct. These coding  
sequences may be arranged in-frame so that subsequent transcription and  
translation is continuous through both coding sequences and results in a

fusion protein. All constructs would have a 5' ATG codon to encode an N-terminal methionine, and a C-terminal translational stop codon.

Thus, a the light chain of a clostridial neurotoxin (or a fragment of the light chain containing the endopeptidase activity) may be expressed recombinantly as a fusion protein with a TM which can also effect the internalisation of the L-chain (or a fragment thereof) into the cytoplasm of the relevant non-neuronal cells responsible for secretion. The expressed fusion protein may also include one or more spacer regions.

In the case of an agent based on clostridial neurotoxin, the following information would be required to produce said agent recombinantly:-

- (i) DNA sequence data relating to a selected TM;
- (ii) DNA sequence data relating to the clostridial neurotoxin component; and
- (iii) a protocol to permit construction and expression of the construct comprising (i) and (ii).

All of the above basic information (i)-(iii) are either readily available, or are readily determinable by conventional methods. For example, both WO98/07864 and WO99/17806 exemplify clostridial neurotoxin recombinant technology suitable for use in the present application.

In addition, methods for the construction and expression of the constructs of the present invention may employ information from the following references and others:-

Lorberboum-Galski, H., FitzGerald, D., Chaudhary, V., Adhya, S., Pastan, I. (1988). Cytotoxic activity of an interleukin 2-Pseudomonas exotoxin chimeric protein produced in Escherichia coli. Proc Natl Acad Sci U S A 85(6):1922-6;

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Murphy, J.R. (1988) Diphtheria-related peptide hormone gene fusions: a molecular genetic approach to chimeric toxin development. *Cancer Treat Res*; 37:123-40;

5 Williams, D.P., Parker, K., Bacha, P., Bishai, W., Borowski, M., Genbauffe, F., Strom, T.B., Murphy, J.R. (1987). Diphtheria toxin receptor binding domain substitution with interleukin-2: genetic construction and properties of a diphtheria toxin-related interleukin-2 fusion protein. *Protein Eng*;1(6):493-8;

10

Arora, N., Williamson, L.C., Leppla, S.H., Halpern, J.L. (1994). Cytotoxic effects of a chimeric protein consisting of tetanus toxin light chain and anthrax toxin lethal factor in non-neuronal cells *J Biol Chem*, 269(42):26165-71;

15

Brinkmann, U., Reiter, Y., Jung, S.H., Lee, B., Pastan, I. (1993). A recombinant immunotoxin containing a disulphide-stabilized Fv fragment. *Proc Natl Acad Sci U S A*;90(16):7538-42; and

20

O'Hare, M., Brown, A.N., Hussain, K., Gebhardt, A., Watson, G., Roberts, L.M., Vitetta, E.S., Thorpe, P.E., Lord, J.M. (1990). Cytotoxicity of a recombinant ricin-A-chain fusion protein containing a proteolytically-cleavable spacer sequence. *FEBS Lett* Oct 29;273(1-2):200-4.

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Suitable clostridial neurotoxin sequence information relating to L- and LH<sub>N</sub>-chains may be obtained from, for example, Kurazono, H. (1992) *J. Biol. Chem.*, vol. 267, No. 21, pp.14721-14729; and Popoff, M.R., and Marvaud, J.-C. (1999) *The Comprehensive Sourcebook of Bacterial Protein Toxins, 2nd edition* (ed. Alouf, J.E., and Freer, J.H.), Academic Press,

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Similarly, suitable TM sequence data are widely available in the art.

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Alternatively, any necessary sequence data may be obtained by techniques which were well-known to the skilled person.

5 For example, DNA encoding the TM component may be cloned from a source organism by screening a cDNA library for the correct coding region (for example by using specific oligonucleotides based on the known sequence information to probe the library), isolating the TM DNA, sequencing this DNA for confirmation purposes, and then placing the isolated DNA in an appropriate expression vector for expression in the  
10 chosen host.

As an alternative to isolation of the sequence from a library, the available sequence information may be employed to prepare specific primers for use in PCR, whereby the coding sequence is then amplified directly from the  
15 source material and, by suitable use of primers, may be cloned directly into an expression vector.

Another alternative method for isolation of the coding sequence is to use the existing sequence information and synthesise a copy, possibly  
20 incorporating alterations, using DNA synthesis technology. For example, DNA sequence data may be generated from existing protein and/or RNA sequence information. Using DNA synthesis technology to do this (and the alternative described above) enables the codon bias of the coding sequence to be modified to be optimal for the chosen expression host. This may give  
25 rise to superior expression levels of the fusion protein.

Optimisation of the codon bias for the expression host may be applied to the DNA sequences encoding the TM and clostridial components of the construct. Optimisation of the codon bias is possible by application of the  
30 protein sequence into freely available DNA/protein database software, eg. programs available from Genetics Computer Group, Inc.

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According to a further aspect of the present invention, nucleic acid encoding the light chain of a clostridial neurotoxin (or a fragment of the light chain containing the endopeptidase activity), may be associated with a TM which can also effect the internalisation of the nucleic acid encoding the L-chain (or a fragment thereof) into the cytoplasm of the relevant non-neuronal cells responsible for secretion. The nucleic acid sequence may be coupled to a translocation domain, and optionally to a targeting moiety, by for example direct covalent linkage or via spacer molecule technology. Ideally, the coding sequence will be expressed in the target cell.

Thus, the agent of the present invention may be the expression product of a recombinant gene delivered independently to the preferred site of action of the agent. Gene delivery technologies are widely reported in the literature [reviewed in "*Advanced Drug Delivery Reviews*" Vol. 27, (1997), Elsevier Science Ireland Ltd].

According to another aspect, the present invention therefore provides a method of treating a condition or disease which is susceptible of treatment with a nucleic acid in a mammal eg. a human which comprises administering to the sufferer an effective, non-toxic amount of a compound of the invention. A condition or disease which is susceptible of treatment with a nucleic acid may be for example a condition or disease which may be treated by or requiring gene therapy. The preferred conditions or diseases susceptible to treatment according to the present invention, together with the preferred TMs, have been described previously in this specification. Similarly, the preferred first domains which cleave one or more proteins (eg. SNAP-25, synaptobrevin and syntaxin) essential to exocytosis have been described previously in this specification. The various domains of an agent for use in gene therapy may be directly linked (eg. via a covalent bond) or indirectly linked (eg. via a spacer molecule), as for example previously described in this specification.

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The invention further provides a compound of the invention for use as an active therapeutic substance, in particular for use in treating a condition or disease as set forth in the present claims.

5       The invention further provides pharmaceutical compositions comprising an agent or a conjugate of the invention and a pharmaceutically acceptable carrier.

10       In use the agent or conjugate will normally be employed in the form of a pharmaceutical composition in association with a human pharmaceutical carrier, diluent and/or excipient, although the exact form of the composition will depend on the mode of administration.

15       The conjugate may, for example, be employed in the form of an aerosol or nebulisable solution for inhalation or a sterile solution for parenteral administration, intra-articular administration or intra-cranial administration.

20       For treating endocrine targets, i.v. injection, direct injection into gland, or aerosolisation for lung delivery are preferred; for treating inflammatory cell targets, i.v. injection, sub-cutaneous injection, or surface patch administration are preferred; for treating exocrine targets, i.v. injection, or direct injection into the gland are preferred; for treating immunological targets, i.v. injection, or injection into specific tissues e.g thymus, bone marrow, or lymph tissue are preferred; for treatment of cardiovascular  
25       targets, i.v. injection is preferred; and for treatment of bone targets, i.v. injection, or direct injection is preferred. In cases of i.v. injection, this should also include the use of pump systems.

30       The dosage ranges for administration of the compounds of the present invention are those to produce the desired therapeutic effect. It will be appreciated that the dosage range required depends on the precise nature of the conjugate, the route of administration, the nature of the formulation,

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the age of the patient, the nature, extent or severity of the patient's condition, contraindications, if any, and the judgement of the attending physician.

5        Suitable daily dosages are in the range 0.0001-1 mg/kg, preferably 0.0001-0.5mg/kg, more preferably 0.002-0.5mg/kg, and particularly preferably 0.004-0.5mg/kg. The unit dosage can vary from less than 1 microgram to 30mg, but typically will be in the region of 0.01 to 1mg per dose, which may be administered daily or less frequently, such as weekly or six  
10       monthly.

Wide variations in the required dosage, however, are to be expected depending on the precise nature of the conjugate, and the differing efficiencies of various routes of administration. For example, oral  
15       administration would be expected to require higher dosages than administration by intravenous injection.

Variations in these dosage levels can be adjusted using standard empirical routines for optimisation, as is well understood in the art.

20       Compositions suitable for injection may be in the form of solutions, suspensions or emulsions, or dry powders which are dissolved or suspended in a suitable vehicle prior to use.

25       Fluid unit dosage forms are typically prepared utilising a pyrogen-free sterile vehicle. The active ingredients, depending on the vehicle and concentration used, can be either dissolved or suspended in the vehicle.

30       Solutions may be used for all forms of parenteral administration, and are particularly used for intravenous injection. In preparing solutions the compound can be dissolved in the vehicle, the solution being made isotonic if necessary by addition of sodium chloride and sterilised by filtration

through a sterile filter using aseptic techniques before filling into suitable sterile vials or ampoules and sealing. Alternatively, if solution stability is adequate, the solution in its sealed containers may be sterilised by autoclaving.

5

Advantageously additives such as buffering, solubilising, stabilising, preservative or bactericidal, suspending or emulsifying agents and/or local anaesthetic agents may be dissolved in the vehicle.

10

Dry powders which are dissolved or suspended in a suitable vehicle prior to use may be prepared by filling pre-sterilised drug substance and other ingredients into a sterile container using aseptic technique in a sterile area.

15

Alternatively the agent and other ingredients may be dissolved in an aqueous vehicle, the solution is sterilized by filtration and distributed into suitable containers using aseptic technique in a sterile area. The product is then freeze dried and the containers are sealed aseptically.

20

Parenteral suspensions, suitable for intramuscular, subcutaneous or intradermal injection, are prepared in substantially the same manner, except that the sterile compound is suspended in the sterile vehicle, instead of being dissolved and sterilisation cannot be accomplished by filtration. The compound may be isolated in a sterile state or alternatively it may be sterilised after isolation, e.g. by gamma irradiation.

25

Advantageously, a suspending agent for example polyvinylpyrrolidone is included in the composition to facilitate uniform distribution of the compound.

30

Compositions suitable for administration via the respiratory tract include aerosols, nebulisable solutions or microfine powders for insufflation. In the latter case, particle size of less than 50 microns, especially less than 10

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microns, is preferred. Such compositions may be made up in a conventional manner and employed in conjunction with conventional administration devices.

5 The agent described in this invention can be used *in vivo*, either directly or as a pharmaceutically acceptable salt, for the treatment of conditions involving secretion from non-neuronal cells, such as hypersecretion of endocrine cell derived chemical messengers, hypersecretion from exocrine cells, secretions from the cells of the immune system, the cardiovascular  
10 system and from bone cells.

The present invention will now be described by reference to the following examples illustrated by the accompanying drawings in which:-

15 Fig. 1 shows SDS-PAGE analysis of WGA-LH<sub>N</sub>/A purification scheme;

Fig. 2 shows activity of WGA-LH<sub>N</sub>/A on release of transmitter from HIT-T15 cells;

20 Fig. 3 shows correlation of SNAP-25 cleavage with inhibition of neurotransmitter release following application of WGA-LH<sub>N</sub>/A to HIT-T15 cells;

25 Fig. 4 shows activity of WGA-LH<sub>N</sub>/A on release of [<sup>3</sup>H]-noradrenaline from undifferentiated PC12 cells;

Fig. 5 shows a Western blot indicating expression of *recLH<sub>N</sub>/B* in *E. coli*;

30 Fig. 6 shows *in vitro* cleavage of synthetic VAMP peptide by *recLH<sub>N</sub>/B*;

- 30 -

- Fig. 7 shows the effect of low pH and BoNT/B treatment on stimulated von Willebrands Factor (vWF) release from human umbilical vein endothelial cells;
- 5 Fig. 8 shows release of [<sup>3</sup>H]-glucosamine labelled high molecular weight material from LS180 cells; and
- 10 Fig. 9 shows the effect of low pH and BoNT/B treatment on stimulated  $\beta$ -glucuronidase release from differentiated HL60 cells.

Figures 5-9 are now described in more detail.

15 Referring to Fig. 5, MBP-LH<sub>N</sub>/B was expressed in *E. coli* as described in Example 4. Lane 1 represents the profile of the expressed fusion protein in *E. coli*. Lane 2 represents the profile of fusion protein expression in the crude *E. coli* lysate. Lane 3 represents the profile of the MBP-LH<sub>N</sub>/B following purification by immobilised amylose. Molecular weights in kDa are indicated to the right side of the Figure.

20 Referring to Fig. 6, dilutions of *rec*LH<sub>N</sub>/B (prepared as described in Example 4) and BoNT/B were compared in an *in vitro* peptide cleavage assay. Data indicate that the recombinant product has similar catalytic activity to that of the native neurotoxin, indicating that the recombinant product has folded correctly into an active conformation.

25

30 Referring to Fig. 7, cells were exposed to pH 4.7 media with or without 500nM BoNT/B (control cells received pH7.4 medium) for 2.5 hours then washed. 24 hours later release of vWF was stimulated using 1mM histamine and the presented results are the net stimulated release with basal subtracted. Results are presented in mIU of vWF/ml and are the mean  $\pm$  SEM of three determinations apart from pH 4.7 alone which is two

determinations. pH 4.7 + BoNT/B has reduced vWF release by 27.4% compared to pH 4.7 controls.

Referring to Fig. 8, high molecular weight mucin synthesising colon carcinoma LS180 cells were treated with pH 4.7 medium and pH 4.7 medium containing 500nM botulinum neurotoxin type B (BoNT/B) for four hours then labelled with [<sup>3</sup>H]-glucosamine for 18 hours. Release of high molecular weight material was stimulated with 10 $\mu$ M ionomycin and [<sup>3</sup>H]-glucosamine labelled material recovered by ultracentrifugation and centrifugal molecular weight sieving. Radiolabel of release of labelled high molecular weight material was determined by scintillation counting and net stimulated release calculated by subtracting non-stimulated basal values. Data are expressed as disintegrations per minute (dpm)  $\pm$  SEM of three determinations. BoNT/B co-treatment clearly inhibits the release of high molecular weight material from these mucin synthesising cells and in this experiment a 74.5% reduction was seen.

Referring to Fig. 9, cells were exposed to pH 4.8 media with or without 500nM BoNT/B (control cells received pH 7.4 medium) for 2.5 hours then washed and differentiated for 40 hours by the addition of 300 $\mu$ M dibutyryl cyclic AMP (dbcAMP). Cells were stimulated with fMet-Leu-Phe (1 $\mu$ M) + ATP (100 $\mu$ M) in the presence of cytochalasin B (5 $\mu$ M) for 10 minutes and released  $\beta$ -glucuronidase determined by colourimetric assay. Net stimulated release was calculated by subtraction of unstimulated basal release values from stimulated values and released activity is expressed as a percentage of the total activity present in the cells. Data are the mean  $\pm$  SEM of three determinations. BoNT/B treatment in low pH medium significantly inhibited stimulated release of  $\beta$ -glucuronidase compared to cells treated with low pH alone ( $p=0.0315$  when subjected to a 2 tailed Student T test with groups of unequal variance).

#### Exempl 1

## **Production of a conjugate of a lectin from *Triticum vulgaris* and LH<sub>N</sub>/A**

### ***Materials***

Lectin from *Triticum vulgaris* (Wheat Germ Agglutinin - WGA) was obtained  
5 from Sigma Ltd.

SPDP was from Pierce Chemical Co.

PD-10 desalting columns were from Pharmacia.

Dimethylsulphoxide (DMSO) was kept anhydrous by storage over a  
molecular sieve.

10 Denaturing sodium dodecylsulphate polyacrylamide gel electrophoresis  
(SDS-PAGE) and non-denaturing polyacrylamide gel electrophoresis was  
performed using gels and reagents from Novex.

Additional reagents were obtained from Sigma Ltd.

15 LH<sub>N</sub>/A was prepared according to a previous method (Shone, C. C. and  
Tranter, H. S. (1995) in "Clostridial Neurotoxins - The molecular  
pathogenesis of tetanus and botulism", (Montecucco, C., Ed.), pp. 152-  
160, Springer). FPLC<sup>®</sup> chromatography media and columns were obtained  
from Amersham Pharmacia Biotech, UK. Affi-gel Hz<sup>™</sup> matrix and materials  
20 were from BioRad, UK.

### ***Preparation of an anti-BoNT/A antibody-affinity column***

An antibody-affinity column was prepared with specific monoclonal  
antibodies essentially as suggested by the manufacturers' protocol. Briefly,  
25 monoclonal antibodies 5BA2.3 & 5BA9.3 which have different epitope  
recognition in the H<sub>C</sub> domain (Hallis, B., Fooks, S., Shone, C. and  
Hambleton, P. (1993) in "Botulinum and Tetanus Neurotoxins", (DasGupta,  
B. R., Ed.), pp. 433-436, Plenum Press, New York) were purified from  
mouse hybridoma tissue culture supernatant by Protein G (Amersham  
30 Pharmacia Biotech) chromatography. These antibodies represent a source  
of BoNT/A H<sub>C</sub>-specific binding molecules and can be immobilised to a  
matrix or used free in solution to bind BoNT/A. In the presence of partially

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purified LH<sub>N</sub>/A (which has no H<sub>C</sub> domain) these antibodies will only bind to BoNT/A. The antibodies 5BA2.3 & 5BA9.3 were pooled in a 3:1 ratio and two mg of the pooled antibody was oxidised by the addition of sodium periodate (final concentration of 0.2%) prior coupling to 1ml Affi-Gel Hz™ gel (16 hours at room temperature). Coupling efficiencies were routinely greater than 65%. The matrix was stored at 4 °C in the presence of 0.02% sodium azide.

*Purification strategy for the preparation of pure LH<sub>N</sub>/A*

BoNT/A was treated with 17µg trypsin per mg BoNT/A for a period of 72-120 hours. After this time no material of 150kDa was observed by SDS-PAGE and Coomassie blue staining. The trypsin digested sample was chromatographed (FPLC® system, Amersham Pharmacia Biotech) on a Mono Q® column (HR5/5) to remove trypsin and separate the majority of BoNT/A from LH<sub>N</sub>/A. The crude sample was loaded onto the column at pH 7 in 20mM HEPES, 50mM NaCl and 2ml LH<sub>N</sub>/A fractions eluted in a NaCl gradient from 50mM to 150mM. The slightly greater pI of BoNT/A (6.3) relative to LH<sub>N</sub>/A (5.2) encouraged any BoNT/A remaining after trypsinisation to elute from the anion exchange column at a lower salt concentration than LH<sub>N</sub>/A. LH<sub>N</sub>/A containing fractions (as identified by SDS-PAGE) were pooled for application to the antibody column.

The semi-purified LH<sub>N</sub>/A mixture was applied and reapplied at least 3 times to a 1-2ml immobilised monoclonal antibody matrix at 20 °C. After a total of 3 hours in contact with the immobilised antibodies, the LH<sub>N</sub>/A-enriched supernatant was removed. Entrapment of the BoNT/A contaminant, rather than specifically binding the LH<sub>N</sub>/A, enables the elution conditions to be maintained at the optimum for LH<sub>N</sub> stability. The use of harsh elution conditions e.g. low pH, high salt, chaotropic ions, which may have detrimental effects on LH<sub>N</sub> polypeptide folding and enzymatic activity, are therefore avoided. Treatment of the immobilised antibody column with 0.2M glycine/HCl pH2.5 resulted in regeneration of the column and elution

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of BoNT/A-reactive proteins of 150kDa.

5 The  $LH_N/A$  enriched sample was then applied 2 times to a 1ml HiTrap<sup>®</sup> Protein G column (Amersham Pharmacia Biotech) at 20 °C. Protein G was selected since it has a high affinity for mouse monoclonal antibodies. This step was included to remove BoNT/A-antibody complexes that may leach from the immunocolumn. Antibody species bind to the Protein G matrix allowing purified  $LH_N/A$  to elute, essentially by the method of Shone C.C., Hambleton, P., and Melling, J. 1987, *Eur. J. Biochem.* 167, 175-180, and  
10 as described in PCT/GB00/03519.

#### *Methods*

The lyophilised lectin was rehydrated in phosphate buffered saline (PBS) to a final concentration of 10 mg/ml. Aliquots of this solution were stored at -  
15 20 °C until use.

The WGA was reacted with an equal concentration of SPDP by the addition of a 10 mM stock solution of SPDP in DMSO with mixing. After one hour at room temperature the reaction was terminated by desalting into PBS  
20 over a PD-10 column.

The thiopyridone leaving group was removed from the product to release a free -SH group by reduction with dithiothreitol (DTT; 5 mM; 30 min). The thiopyridone and DTT were removed by once again desalting into PBS over  
25 a PD-10 column.

The  $LH_N/A$  was desalted into PBSE (PBS containing 1 mM EDTA). The resulting solution (0.5-1.0 mg/ml) was reacted with a four-fold molar excess of SPDP by addition of a 10 mM stock solution of SPDP in DMSO.  
30 After 3 h at room temperature the reaction was terminated by desalting over a PD-10 column into PBSE.

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A portion of the derivatized LH<sub>N</sub>/A was removed from the solution and reduced with DTT (5 mM, 30 min). This sample was analyzed spectrophotometrically at 280 nm and 343 nm to determine the degree of derivatisation. The degree of derivatisation achieved was  $3.53 \pm 0.59$  mol/mol.

The bulk of the derivatized LH<sub>N</sub>/A and the derivatized WGA were mixed in proportions such that the WGA was in greater than three-fold molar excess. The conjugation reaction was allowed to proceed for > 16 h at 4 °C.

The product mixture was centrifuged to clear any precipitate that had developed. The supernatant was concentrated by centrifugation through concentrators (with 10000 molecular weight exclusion limit) before application to a Superose 12 column on an FPLC chromatography system (Pharmacia). The column was eluted with PBS and the elution profile followed at 280 nm.

Fractions were analyzed by SDS-PAGE on 4-20% polyacrylamide gradient gels, followed by staining with Coomassie Blue. The major conjugate products have an apparent molecular mass of between 106-150 kDa, these are separated from the bulk of the remaining unconjugated LH<sub>N</sub>/A and more completely from the unconjugated WGA. Fractions containing conjugate were pooled prior to addition to PBS-washed N-acetylglucosamine-agarose. Lectin-containing proteins (i.e. WGA-LH<sub>N</sub>/A conjugate) remained bound to the agarose during washing with PBS to remove contaminants (predominantly unconjugated LH<sub>N</sub>/A). WGA-LH<sub>N</sub>/A conjugate was eluted from the column by the addition of 0.3M N-acetylglucosamine (in PBS) and the elution profile followed at 280 nm. See Fig 1 for SDS-PAGE profile of the whole purification scheme.

The fractions containing conjugate were pooled, dialysed against PBS, and

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stored at 4 °C until use.

## **Example 2**

### **Activity of WGA-LH<sub>N</sub>/A in cultured endocrine cells (HIT-T15)**

5

The hamster pancreatic B cell line HIT-T15 is an example of a cell line of endocrine origin. It thus represents a model cell line for the investigation of inhibition of release effects of the agents. HIT-T15 cells possess surface moieties that allow for the binding, and internalisation, of WGA-LH<sub>N</sub>/A.

10

In contrast, HIT-T15 cells lack suitable receptors for clostridial neurotoxins and are therefore not susceptible to botulinum neurotoxins (BoNTs).

15

Fig. 2 illustrates the inhibition of release of insulin from HIT-T15 cells after prior incubation with WGA-LH<sub>N</sub>/A. It is clear that dose-dependent inhibition is observed, indicating that WGA-LH<sub>N</sub>/A can inhibit the release of insulin from an endocrine cell model.

20

Inhibition of insulin release was demonstrated to correlate with cleavage of the SNARE protein, SNAP-25 (Fig. 3). Thus, inhibition of release of chemical messenger is due to a clostridial endopeptidase-mediated effects of SNARE-protein cleavage.

### ***Materials***

25

Insulin radioimmunoassay kits were obtained from Linco Research Inc., USA.

Western blotting reagents were obtained from Novex.

### ***Methods***

30

HIT-T15 cells were seeded onto 12 well plates and cultured in RPMI-1640 medium containing 5% foetal bovine serum, 2mM L-glutamine for 5 days prior to use. WGA-LH<sub>N</sub>/A was applied for 4 hours on ice, the cells were

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washed to remove unbound WGA-LH<sub>N</sub>/A, and the release of insulin assayed 16 hours later. The release of insulin from HIT-T15 cells was assessed by radioimmunoassay exactly as indicated by the manufacturers' instructions.

5 Cells were lysed in 2M acetic acid / 0.1% TFA. Lysates were dried then resuspended in 0.1M Hepes, pH 7.0. To extract the membrane proteins Triton-X-114 (10%, v/v) was added and incubated at 4°C for 60 min. The insoluble material was removed by centrifugation and the supernatants were warmed to 37°C for 30 min. The resulting two phases were separated  
10 by centrifugation and the upper phase discarded. The proteins in the lower phase were precipitated with chloroform/methanol for analysis by Western blotting.

The samples were separated by SDS-PAGE and transferred to  
15 nitrocellulose. Proteolysis of SNAP-25, a crucial component of the neurosecretory process and the substrate for the zinc-dependent endopeptidase activity of BoNT/A, was then detected by probing with an antibody (SMI-81) that recognises both the intact and cleaved forms of SNAP-25.

20

### **Example 3**

#### **Activity of WGA-LH<sub>N</sub>/A in cultured neuroendocrine cells (PC12)**

The rat pheochromocytoma PC12 cell line is an example of a cell line of  
25 neuroendocrine origin. In its undifferentiated form it has properties associated with the adrenal chromaffin cell [Greene and Tischler, in "*Advances in Cellular Neurobiology*" (Federoff and Hertz, eds), Vol. 3, p373-414. Academic Press, New York, 1982]. It thus represents a model cell line for the investigation of inhibition of release effects of the agents.  
30 PC12 cells possess surface moieties that allow for the binding, and internalisation, of WGA-LH<sub>N</sub>/A. Figure 4 illustrates the inhibition of release of noradrenaline from PC12 cells after prior incubation with WGA-LH<sub>N</sub>/A.

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It is clear that dose-dependent inhibition is observed, indicating that WGA-LH<sub>N</sub>/A can inhibit the release of hormone from a neuroendocrine cell model. Comparison of the inhibition effects observed with conjugate and the untargeted LH<sub>N</sub>/A demonstrate the requirement for a targeting moiety (TM) for efficient inhibition of transmitter release.

#### *Methods*

PC12 cells were cultured on 24 well plates in RPMI-1640 medium containing 10% horse serum, 5% foetal bovine serum, 1% L-glutamine. Cells were treated with a range of concentrations of WGA-LH<sub>N</sub>/A for three days. Secretion of noradrenaline was measured by labelling cells with [<sup>3</sup>H]-noradrenaline (2μCi/ml, 0.5ml/well) for 60 min. Cells were washed every 15 min for 1 hour then basal release determined by incubation with a balanced salt solution containing 5mM KCl for 5 min. Secretion was stimulated by elevating the concentration of extracellular potassium (100mM KCl) for 5 min. Radioactivity in basal and stimulated superfusates was determined by scintillation counting. Secretion was expressed as a percentage of the total uptake and stimulated secretion was calculated by subtracting basal. Inhibition of secretion was dose-dependent with an observed IC<sub>50</sub> of 0.63 ± 0.15μg/ml (n = 3). Inhibition was significantly more potent when compared to untargeted endopeptidase (LH<sub>N</sub>/A in Fig. 4). Thus WGA-LH<sub>N</sub>/A inhibits release of neurotransmitter from a model neuroendocrine cell type.

#### **Example 4**

##### **Expression and purification of catalytically active recombinant LH<sub>N</sub>/B**

The coding region for LH<sub>N</sub>/B was inserted in-frame to the 3' of the gene encoding maltose binding protein (MBP) in the expression vector pMAL (New England Biolabs). In this construct, the expressed MBP and LH<sub>N</sub>/B polypeptides are separated by a Factor Xa cleavage site.

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Expression of the MBP-LH<sub>N</sub>/B in *E. coli* TG1 was induced by addition of IPTG to the growing culture at an approximate OD<sub>600nm</sub> of 0.8. Expression was maintained for a further 3 hours in the presence of inducing agent prior to harvest by centrifugation. The recovered cell paste was  
5 stored at -20 °C until required.

The cell paste was resuspended in resuspension buffer (50mM Hepes pH7.5 + 150mM NaCl + a variety of protease inhibitors) at 6ml buffer per gram paste. To this suspension was added lysozyme to a final  
10 concentration of 1mg/ml. After 10 min at 0 °C, the suspension was sonicated for 6x30 seconds at 24μ at 0 °C. The broken cell paste was then centrifuged to remove cell debris and the supernatant recovered for chromatography.

15 In some situations, the cell paste was disrupted by using proprietary disruption agents such as BugBuster™ (Novagen) as per the manufacturers protocol. These agents were satisfactory for disruption of the cells to provide supernatant material for affinity chromatography.

20 The supernatant was applied to an immobilised amylose matrix at 0.4ml/min to facilitate binding of the fusion protein. After binding, the column was washed extensively with resuspension buffer to remove contaminating proteins. Bound proteins were eluted by the addition of elution buffer (resuspension buffer + 10mM maltose) and fractions  
25 collected. Eluted fractions containing protein were pooled for treatment with Factor Xa.

On some occasions a further purification step was incorporated into the scheme, prior to the addition of Factor Xa. In these instances, the eluted  
30 fractions were made to 5mM DTT and applied to a Pharmacia Mono-Q HR5/5 column (equilibrated in resuspension buffer) as part of an FPLC system. Proteins were bound to the column at 150mM NaCl, before

- 40 -

increased to 500mM NaCl over a gradient. Fractions were collected and analysed for the presence of MBP-LH<sub>N</sub>/B by Western blotting (probe antibody = guinea pig anti-BoNT/B or commercially obtained anti-MBP).

5      Cleavage of the fusion protein by Factor Xa was as described in the protocol supplied by the manufacturer (New England Biolabs). Cleavage of the fusion protein resulted in removal of the MBP fusion tag and separation of the LC and H<sub>N</sub> domains of LH<sub>N</sub>/B. Passage of the cleaved mixture through a second immobilised maltose column removed free MBP from the mixture to leave purified disulphide-linked LH<sub>N</sub>/B. This material was used for conjugation.

See Figure 5 for an illustration of the purification of LH<sub>N</sub>/B.

See Figure 6 for an illustration of the *in vitro* catalytic activity of LH<sub>N</sub>/B.

15

#### **Example 5**

##### **Production of a conjugate of a lectin from *Triticum vulgaris* and LH<sub>N</sub>/B**

#### **Materials**

Lectin from *Triticum vulgaris* (WGA) was obtained from Sigma Ltd.

LH<sub>N</sub>/B was prepared as described in Example 4.

SPDP was from Pierce Chemical Co.

PD-10 desalting columns were from Pharmacia.

25      Dimethylsulphoxide (DMSO) was kept anhydrous by storage over a molecular sieve.

Polyacrylamide gel electrophoresis was performed using gels and reagents from Novex.

Additional reagents were obtained from Sigma Ltd.

30

#### **Methods**

The lyophilised lectin was rehydrated in phosphate buffered saline (PBS) to

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a final concentration of 10 mg/ml. Aliquots of this solution were stored at -20 °C until use.

5       The WGA was reacted with an equal concentration of SPDP by the addition of a 10 mM stock solution of SPDP in DMSO with mixing. After one hour at room temperature the reaction was terminated by desalting into PBS over a PD-10 column.

10       The thiopyridone leaving group was removed from the product to release a free -SH group by reduction with dithiothreitol (DTT; 5 mM; 30 min). The thiopyridone and DTT were removed by once again desalting into PBS over a PD-10 column.

15       The *recLH<sub>N</sub>/B* was desalted into PBS. The resulting solution (0.5-1.0 mg/ml) was reacted with a four-fold molar excess of SPDP by addition of a 10 mM stock solution of SPDP in DMSO. After 3 h at room temperature the reaction was terminated by desalting over a PD-10 column into PBS.

20       A portion of the derivatized *recLH<sub>N</sub>/B* was removed from the solution and reduced with DTT (5 mM, 30 min). This sample was analysed spectrophotometrically at 280 nm and 343 nm to determine the degree of derivatisation.

25       The bulk of the derivatized *recLH<sub>N</sub>/B* and the derivatized WGA were mixed in proportions such that the WGA was in greater than three-fold molar excess. The conjugation reaction was allowed to proceed for > 16 h at 4 °C.

30       The product mixture was centrifuged to clear any precipitate that had developed. The supernatant was concentrated by centrifugation through concentrators (with 10000 molecular weight exclusion limit) before application to a Superdex G-200 column on an FPLC chromatography

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system (Pharmacia). The column was eluted with PBS and the elution profile followed at 280 nm.

Fractions were analysed by SDS-PAGE on 4-20% polyacrylamide gradient gels, followed by staining with Coomassie Blue. The major conjugate products have an apparent molecular mass of between 106-150 kDa, these are separated from the bulk of the remaining unconjugated *recLH<sub>N</sub>/B* and more completely from the unconjugated WGA. Fractions containing conjugate were pooled prior to addition to PBS-washed N-acetylglucosamine-agarose. Lectin-containing proteins (i.e. WGA-*recLH<sub>N</sub>/B* conjugate) remained bound to the agarose during washing with PBS to remove contaminants (predominantly unconjugated *recLH<sub>N</sub>/B*). WGA-*recLH<sub>N</sub>/B* conjugate was eluted from the column by the addition of 0.3M N-acetylglucosamine (in PBS) and the elution profile followed at 280 nm.

The fractions containing conjugate were pooled, dialysed against PBS, and stored at 4 °C until use.

### Example 6

#### Activity of BoNT/B in vascular endothelial cells

Human umbilical vein endothelial cells (HUVEC) secrete von Willebrands Factor (vWF) when stimulated with a variety of cell surface receptor agonists including histamine. These cells maintain this property when prepared from full term umbilical cords and grown in culture (Loesberg et al 1983, Biochim. Biophys. Acta. 763, 160-168). The release of vWF by HUVEC thus represents a secretory activity of a non-neuronal cell type derived from the cardiovascular system. Fig. 7 illustrates the inhibition of the histamine stimulated release of vWF by HUVEC when previously treated with BoNT/B in low pH medium. Treatment of cells with toxins in low pH can be used as a technique for facilitating toxin penetration of the plasmalemma of cells refractory to exogenously applied clostridial

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neurotoxins.

This result clearly shows the ability of botulinum neurotoxins to inhibit secretory activity of non-neuronal cells in the cardiovascular system (see Fig. 7).

### *Methods*

HUVEC were prepared by the method of Jaffe et al 1973, *J. Clin. Invest.* 52, 2745-2756. Cells were passaged once onto 24 well plates in medium 199 supplemented with 10% foetal calf serum, 10% newborn calf serum, 5mM L-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, 20µg/ml endothelial cell growth factor (Sigma). Cells were treated with DMEM pH 7.4, DMEM pH 4.7 (pH lowered with HCl) or DMEM, pH 4.7 with 500nM BoNT/B for 2.5 hours then washed three times with HUVEC medium. 24 hours later cells were washed with a balanced salt solution, pH 7.4 and exposed to this solution for 30 minutes for the establishment of basal release. This was removed and BSS containing 1mM histamine applied for a further 30 minutes. Superfusates were centrifuged to remove any detached cells and the quantity of vWF determined using an ELISA assay as described by Paleolog et al 1990, *Blood*. 75, 688-695. Stimulated secretion was then calculated by subtracting basal from the histamine stimulated release. Inhibition by BoNT/B treatment at pH 4.7 was calculated at 27.4% when compared to pH 4.7 treatment alone.

### **Example 7**

#### **Activity of BoNT/B in mucus secreting cells**

The LS180 colon carcinoma cell line is recognised as a model of mucin secreting cells (McCool, D. J., Forstner, J. F. and Forstner, G. G. 1994 *Biochem. J.* 302, 111-118). These cells have been shown to adopt goblet cell morphology and release high molecular weight mucin when stimulated with muscarinic agonists (eg carbachol), phorbol esters (PMA) and  $Ca^{2+}$

ionophores (eg A23187) (McCool, D. J., Forstner, J. F. and Forstner, G. G. 1995 *Biochem. J.* 312, 125-133). These cells thus represent a non-neuronal cell type derived from the colon which can undergo regulated mucin secretion. Fig. 8 illustrates the inhibition of the ionomycin stimulated release of high molecular weight, [<sup>3</sup>H]-glucosamine labelled material from LS180 cells by pretreatment with BoNT/B in low pH medium. Ionomycin is a Ca<sup>2+</sup> ionophore and treatment of cells with low pH medium has been previously shown to facilitate toxin entry into cells.

This result clearly shows the ability of botulinum neurotoxins to inhibit secretory activity of non-neuronal cells able to release mucin when stimulated with a secretagogue (see Fig. 8).

#### *Methods*

Mucin synthesising colon carcinoma LS180 cells were grown on Matrigel coated 24 well plates in minimum essential medium supplemented with 10% foetal calf serum, 2mM L-glutamine and 1% non-essential amino acids (Sigma) Cells were treated with pH 7.4 medium, pH 4.7 medium and pH 4.7 medium containing 500nM botulinum neurotoxin type B (BoNT/B) for four hours then labelled with [<sup>3</sup>H]-glucosamine (1μCi/ml, 0.5ml/well) for 18 hours in L15 glucose free medium. Cells were then washed twice with a balanced salt solution (BSS) pH 7.4 and then 0.5ml of BSS was applied for 30 minutes. This material was removed and 0.5ml of BSS containing 10μM ionomycin applied to stimulate mucin release. The stimulating solution was removed and all superfusates centrifuged to remove any detached cells. Supernatants were then centrifuged at 100,000 x g for 1 hour. Supernatants were applied to Centricon centrifugal concentrators with a molecular weight cut-off of 100kDa and centrifuged (2,500 x g) until all liquid had passed through the membrane. Membranes were washed with BSS by centrifugation three times and then the membrane scintillation counted for retained, [<sup>3</sup>H]-glucosamine labelled high molecular weight material.

**Example 8****Activity of BoNT/B in inflammatory cells**

5 The promyelocytic cell line HL60 can be differentiated into neutrophil like cells by the addition of dibutyryl cyclic AMP to the culture medium. Upon differentiation these cells increase their expression of characteristic enzymes such as  $\beta$ -glucuronidase. In this condition these cells therefore represent a model of a phagocytic cell type which contributes to the inflammatory response of certain disease states (eg rheumatoid arthritis).  
10 Figure 9 illustrates the significant ( $p > 0.05$ ) inhibition of stimulated release of  $\beta$ -glucuronidase from dbcAMP differentiated HL60 cells by pre-treatment with BoNT/B in low pH medium.

15 This result clearly shows the ability of botulinum neurotoxins to inhibit the secretory activity of a non-neuronal cell type which is a model of the neutrophil a cell which participates in inflammation.

***Methods***

20 HL60 cells were cultured in RPMI 1640 medium containing 10% foetal calf serum and 2mM glutamine. Cells were exposed to low pH and toxin for 2.5 hours then washed 3 times and differentiated by the addition of dibutyryl cyclic AMP (dbcAMP) to a final concentration of 300 $\mu$ M. Cells were differentiated for 40 hours and then stimulated release of  $\beta$ -glucuronidase activity was determined. Cells were treated with cytochalasin B (5 $\mu$ M) 25 minutes before stimulation. Cells were stimulated with 1 $\mu$ M N-formyl-Met-Leu-Phe with 100 $\mu$ M ATP for 10 minutes then centrifuged and the supernatant taken for assay of  $\beta$ -glucuronidase activity. Activity was measured in cell lysates and the amount released expressed as a percentage of the total cellular content of enzyme.

30

$\beta$ -glucuronidase activity was determined according to the method of Absolom D.R. 1986, (Methods in Enzymology, 132, 160) using *p*-

Nitrophenyl- $\beta$ -D-glucuronide as the substrate.

**CLAIMS**

1. A method of inhibiting secretion from a non-neuronal cell comprising  
5 administering an agent comprising at least first and second domains,  
wherein the first domain cleaves one or more proteins essential to  
exocytosis and the second domain translocates the first domain into the  
cell.
- 10 2. A method according to Claim 1, for treatment of disease caused,  
exacerbated or maintained by secretion from a non-neuronal cell or non-  
neuronal cells.
- 15 3. A method according to Claim 1 or 2, wherein the agent further  
comprises a third domain for targeting the agent to a non-neuronal cell.
4. A method according to Claim 3 wherein the third domain targets the  
agent to an endocrine cell.
- 20 5. A method according to Claim 4 wherein the third domain comprises  
or consists of a ligand selected from iodine; thyroid stimulating hormone  
(TSH); TSH receptor antibodies; antibodies to the islet-specific monosialo-  
ganglioside GM2-1; insulin, insulin-like growth factor and antibodies to the  
25 receptors of both; TSH releasing hormone (protirelin) and antibodies to its  
receptor; FSH/LH releasing hormone (gonadorelin) and antibodies to its  
receptor; corticotrophin releasing hormone (CRH) and antibodies to its  
receptor; and ACTH and antibodies to its receptor.
- 30 6. A method according to Claim 4 or 5 for the treatment of a disease  
caused, exacerbated, or maintained by secretion from an endocrine cell,  
preferably for treatment of a disease selected from endocrine neoplasia  
including MEN; thyrotoxicosis and other diseases dependent on

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hypersecretions from the thyroid; acromegaly, hyperprolactinaemia, Cushings disease and other diseases dependent on anterior pituitary hypersecretion; hyperandrogenism, chronic anovulation and other diseases associated with polycystic ovarian syndrome.

5

7. A method according to Claim 3 wherein the third domain targets the agent to inflammatory cells

10

8. A method according to Claim 7 wherein the third domain comprises or consists of a ligand selected from (i) for mast cells, complement receptors in general, including C4 domain of the Fc IgE, and antibodies/ligands to the C3a/C4a-R complement receptor; (ii) for eosinophils, antibodies/ligands to the C3a/C4a-R complement receptor, anti VLA-4 monoclonal antibody, anti-IL5 receptor, antigens or antibodies reactive toward CR4 complement receptor; (iii) for macrophages and monocytes, macrophage stimulating factor, (iv) for macrophages, monocytes and neutrophils, bacterial LPS and yeast B-glucans which bind to CR3, (v) for neutrophils, antibody to OX42, an antigen associated with the iC3b complement receptor, or IL8; (vi) for fibroblasts, mannose 6-phosphate/insulin-like growth factor-beta (M6P/IGF-II) receptor and PA2.26, antibody to a cell-surface receptor for active fibroblasts in mice.

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9. A method according to Claim 7 or 8 for the treatment of a disease caused, exacerbated, or maintained by secretion from an inflammatory cell, preferably for treatment of a disease selected from allergies (seasonal allergic rhinitis (hay fever), allergic conjunctivitis, vasomotor rhinitis and food allergy), eosinophilia, asthma, rheumatoid arthritis, systemic lupus erythematosus, discoid lupus erythematosus, ulcerative colitis, Crohn's disease, haemorrhoids, pruritus, glomerulonephritis, hepatitis, pancreatitis, gastritis, vasculitis, myocarditis, psoriasis, eczema, chronic radiation-induced fibrosis, lung scarring and other fibrotic disorders.

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10. A method according to Claim 3 wherein the third domain targets the agent to an exocrine cell.

5 11. A method according to Claim 10 wherein the third domain comprises or consists of a ligand selected from pituitary adenyl cyclase activating peptide (PACAP-38) and an antibody to its receptor.

10 12. A method according to Claim 10 or 11 for the treatment of a disease caused, exacerbated, or maintained by secretion from an exocrine cell, preferably for treatment of acute pancreatitis, or for treatment of mucus hypersecretion from mucus-secreting cells of the alimentary tract, in particular from mucus-secreting cells of the colon.

15 13. A method according to Claim 3 wherein the third domain targets the agent to immunological cells.

20 14. A method according to Claim 13 wherein the third domain comprises or consists of a ligand selected from Epstein Barr virus fragment/surface feature and idiotypic antibody (binds to CR2 receptor on B-lymphocytes and lymph node follicular dendritic cells).

25 15. A method according to Claim 13 or 14 for the treatment of a disease caused, exacerbated, or maintained by secretion from an immunological cell, preferably for treatment of a disease selected from myasthenia gravis, rheumatoid arthritis, systemic lupus erythematosus, discoid lupus erythematosus, organ transplant, tissue transplant, fluid transplant, Graves disease, thyrotoxicosis, autoimmune diabetes, haemolytic anaemia, thrombocytopenic purpura, neutropenia, chronic autoimmune hepatitis, autoimmune gastritis, pernicious anaemia, Hashimoto's thyroiditis, 30 Addison's disease, Sjogren's syndrome, primary biliary cirrhosis, polymyositis, scleroderma, systemic sclerosis, pemphigus vulgaris, bullous pemphigoid, myocarditis, rheumatic carditis, glomerulonephritis

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(Goodpasture type), uveitis, orchitis, ulcerative colitis, vasculitis, atrophic gastritis, pernicious anaemia, and type 1 diabetes mellitus.

5 16. A method according to Claim 3 wherein the third domain targets the agent to cells of the cardiovascular system.

10 17. A method according to Claim 16 wherein the third domain comprises or consists of a ligand selected from ligands for targeting platelets, preferably thrombin or TRAP (thrombin receptor agonist peptide), or antibodies to CD31/PECAM-1, CD24 or CD106/VCAM-1, and ligands for targeting cardiovascular endothelial cells, preferably GP1b surface antigen recognising antibodies.

15 18. A method according to Claim 16 or 17 for the treatment of a disease caused, exacerbated or maintained by secretion from a cell of the cardiovascular system, preferably for treatment of disease states involving inappropriate platelet activation and/or thrombus formation, or for treatment of hypertension.

20 19. A method according to Claim 3 wherein the third domain targets the agent to a cell whose secretions can lead to bone disorders.

25 20. A method according to Claim 19 wherein the third domain comprises or consists of a ligand selected from the group consisting of, ligands for targeting osteoblasts, preferably calcitonin, and ligands for targeting osteoclasts, preferably osteoclast differentiation factor (TRANCE, or RANKL or OPGL) or an antibody to the receptor RANK.

30 21. A method according to Claim 19 or 20 for the treatment of a disease caused, exacerbated or maintained by secretion from a cell whose secretions can lead to bone disorders, preferably for the treatment of a disease selected from osteopetrosis and osteoporosis.

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22. A method according to any previous Claim, wherein the agent comprises a first domain that cleaves a protein selected from SNAP-25, synaptobrevin and syntaxin.

5 23. A method according to Claim 22 wherein the first domain comprises a light chain of a clostridial neurotoxin, or a fragment, variant or derivative thereof which inhibits exocytosis.

10 24. A method according to any previous Claim, wherein the second domain comprises a H<sub>N</sub> region of a clostridial polypeptide, or a fragment, variant or derivative thereof that translocates the exocytosis inhibiting activity of the first domain into the cell.

15 25. A method according to any previous Claim for inhibition of constitutive and regulated release from non-neuronal cells.

20 26. An agent for inhibiting secretion from a non-neuronal cell, comprising at least first, second and third domains, wherein the first domain cleaves one or more proteins essential to exocytosis, the second domain translocates the first domain into the cell and the third domain binds to a non-neuronal cell.

25 27. An agent according to Claim 26, wherein the third domain is as defined in any of Claims 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, and 20.

28. A pharmaceutical composition comprising an agent according to Claim 26 or 27 in combination with a pharmaceutically acceptable carrier.

30 29. Use of an agent according to Claim 26 or 27 in treatment of a disease caused, exacerbated or maintained by secretion from a non-neuronal cell.

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30. Use of an agent according to Claim 26 or 27 in manufacture of a medicament for treatment of a disease caused, exacerbated or maintained by secretion from a non-neuronal cell.

5 31. A nucleic acid construct encoding an agent according to Claim 26 or 27, said construct comprising nucleic acid sequences encoding the first, second and third domains.

10 32. A nucleic acid construct according to Claim 31, operably linked to promoter and terminator sequences, and optionally regulatory sequences, said promoter, terminator and regulatory sequences being functional in a target cell to effect expression of said agent in said target cell.

15 33. An agent for use in gene therapy, comprising a nucleic acid sequence encoding a first domain which cleaves one or more proteins essential to exocytosis, and a second domain associated with the nucleic acid sequence which, following administration to a patient, translocates the nucleic acid sequence into a non-neuronal target cell and, when in said non-neuronal target cell, expression of the nucleic acid sequence is effected  
20 therein.

34. An agent according to Claim 33, wherein the nucleic acid sequence is operably linked to promoter and terminator sequences, and optionally regulatory sequences, said promoter, terminator and regulatory sequences  
25 being functional in the non-neuronal target cell to effect expression of said agent in said non-neuronal target cell.

35. An agent according to Claim 32 or 33, wherein the agent further comprises a third domain for targeting the agent to non-neuronal cell.

30

36. A method of treating by gene therapy a disease caused, exacerbated or maintained by secretion from a non-neuronal cell, said method

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comprising administering to a patient an agent according to any of Claims 33-35.

5        37.    Use of a nucleic acid construct according to Claims 31 or 32, or an agent according to any of Claims 33-35, in the manufacture of a medicament for treating by gene therapy a disease caused by, exacerbated, or maintained by secretion from a non-neuronal cell.

10       38.    A method of treating a disease caused, exacerbated or maintained by secretion from a non-neuronal cell, said method comprising administering to a patient a polypeptide that cleaves one or more proteins essential to exocytosis, or a nucleic acid encoding said polypeptide, to a patient.

15       39.    Use of a polypeptide that cleaves one or more proteins essential to exocytosis, or a nucleic acid encoding said polypeptide, in the manufacture of an agent for treating a disease caused by, exacerbated or maintained by secretion from a non-neuronal cell.

**ABSTRACT**

5 A method of treatment of disease by inhibition of cellular secretory  
processes is provided. The method has particular application in the  
treatment of diseases dependent upon the exocytotic activity of endocrine  
cells, exocrine cells, inflammatory cells, cells of the immune system, cells  
of the cardiovascular system, and bone cells. Agents and compositions  
therefor, as well as methods for manufacturing these agents and  
10 compositions, are provided. In a preferred embodiment a clostridial  
neurotoxin, substantially devoid of holotoxin binding affinity for neuronal  
cells of the presynaptic muscular junction, is associated with a targeting  
moiety. The targeting moiety is selected such that the clostridial toxin  
conjugate so formed may be directed to a non-neuronal target cell to which  
15 the conjugate may bind. Following binding, a neurotoxin component of the  
conjugate, which is capable of inhibition of cellular secretion, passes into  
the cytosol of the target cell by cellular internalisation mechanisms.  
Thereafter, inhibition of secretion from the target cell is effected.

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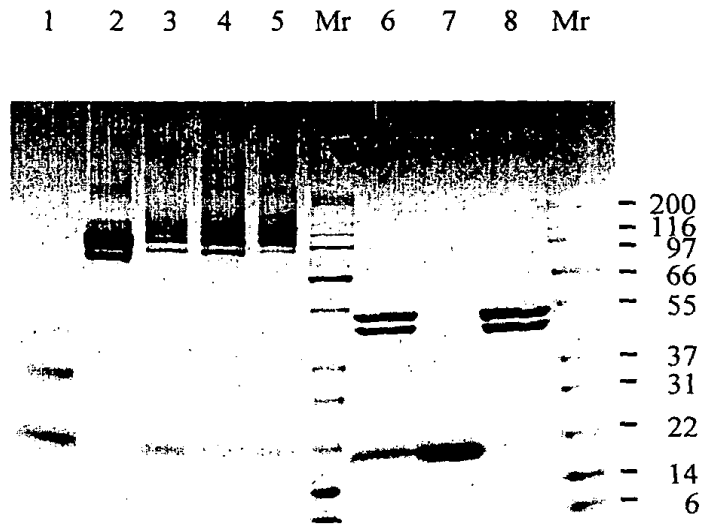
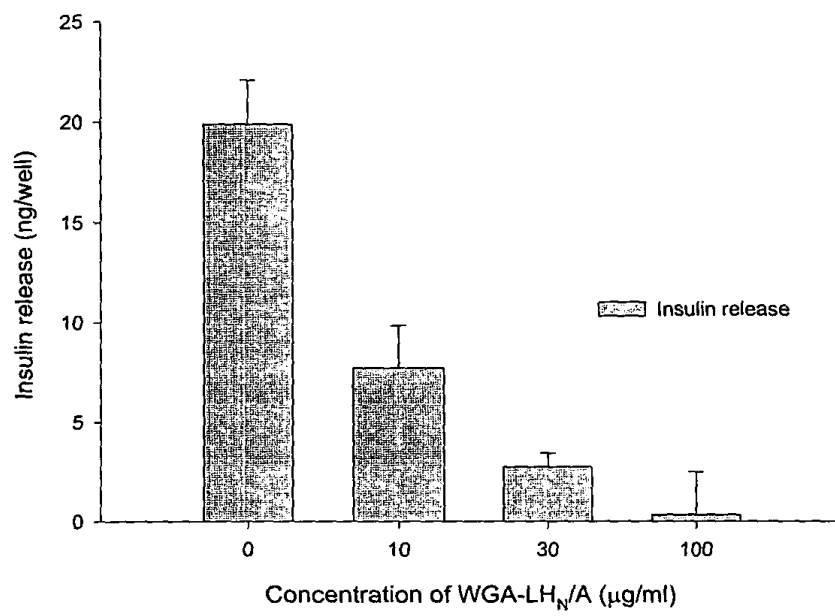


FIG. 1

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**FIG. 2**

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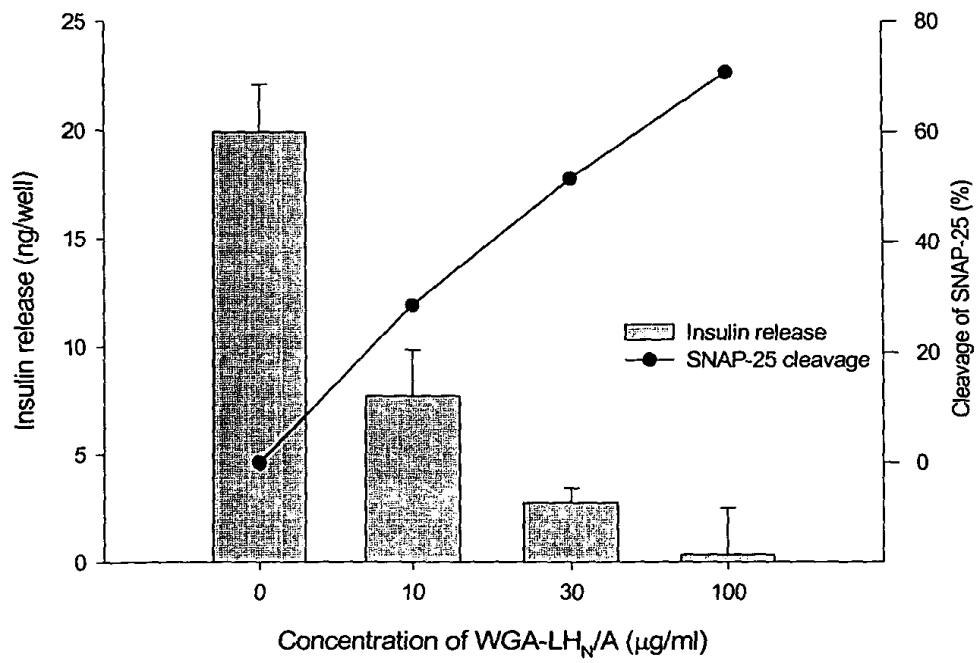


FIG. 3

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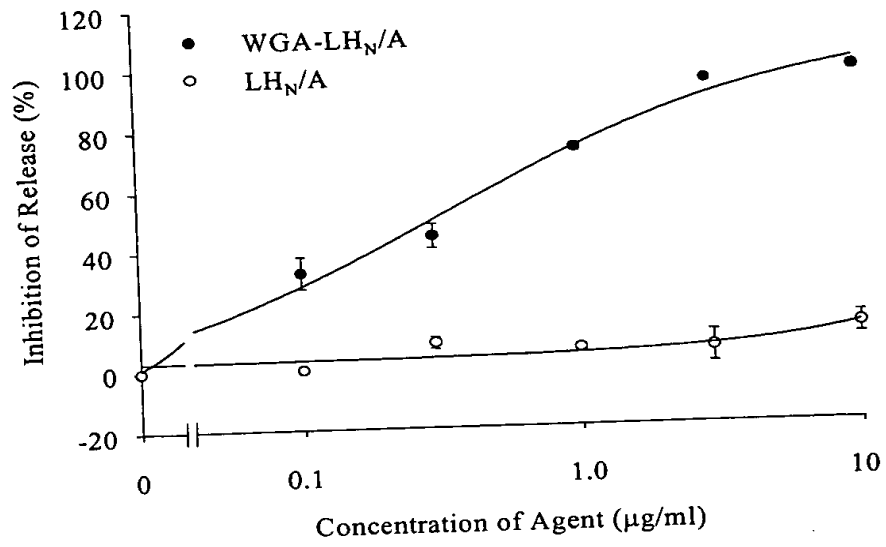


FIG. 4

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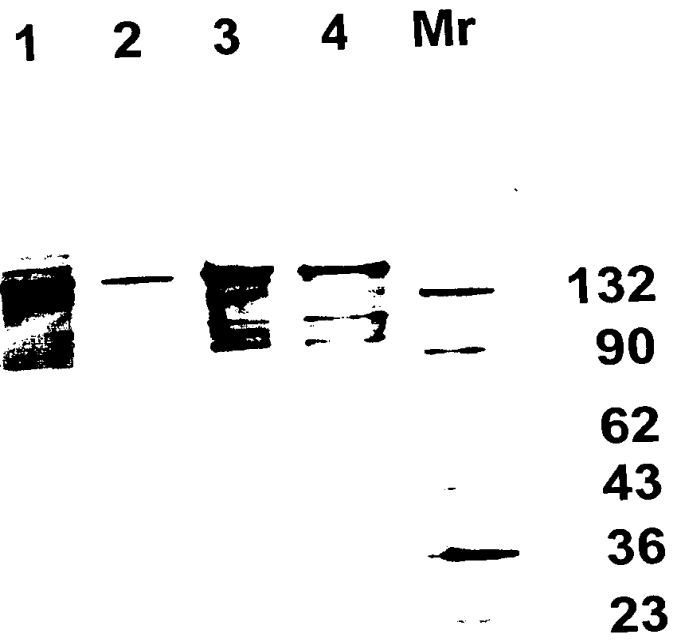


Fig. 5.

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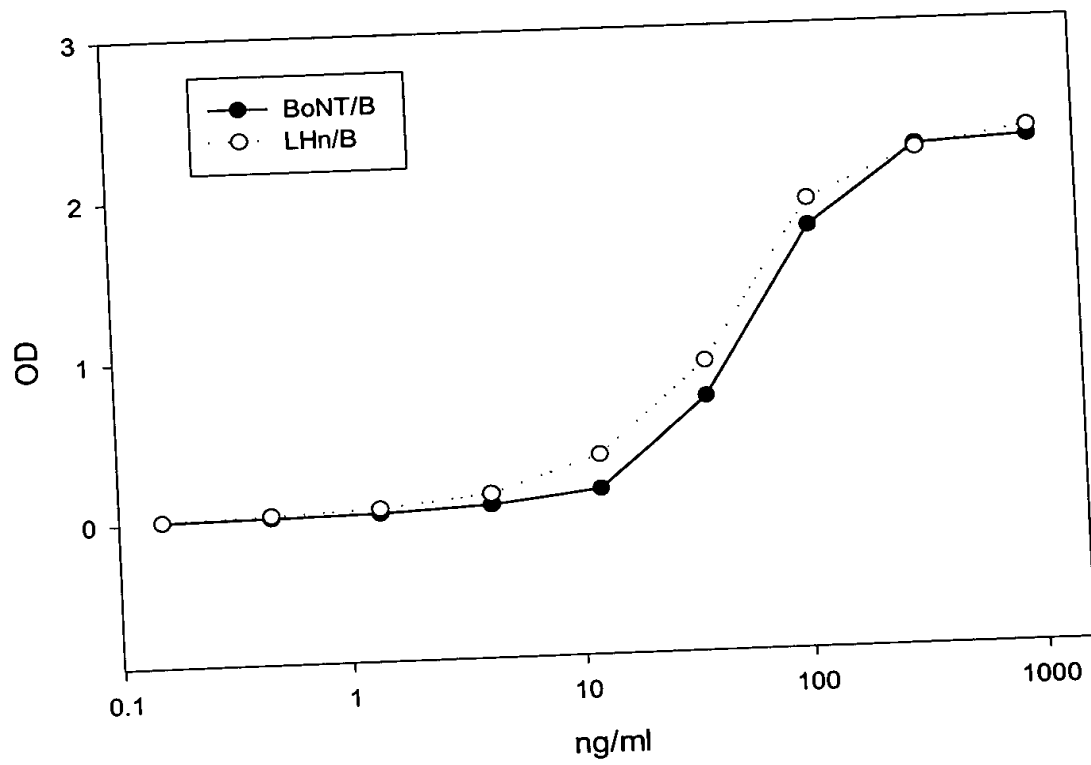


Fig. 6

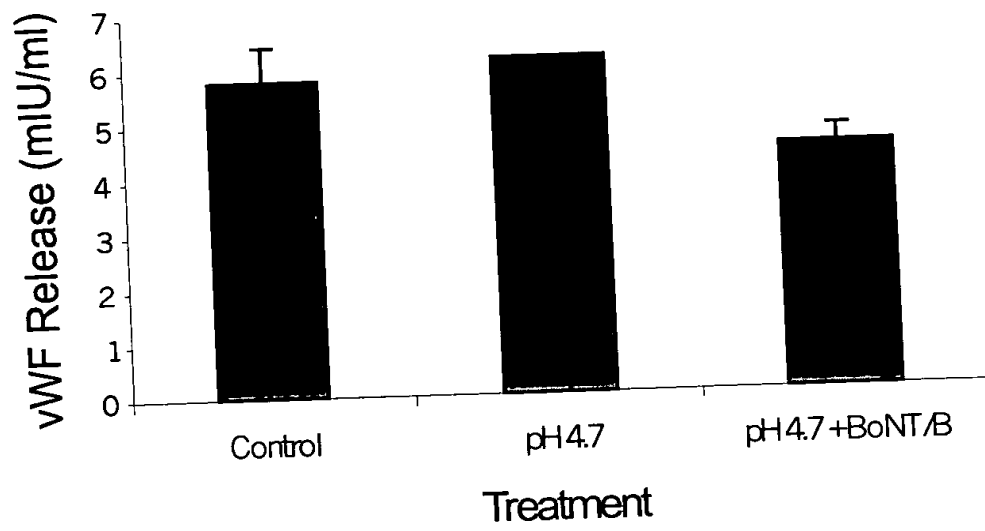


Fig. 7

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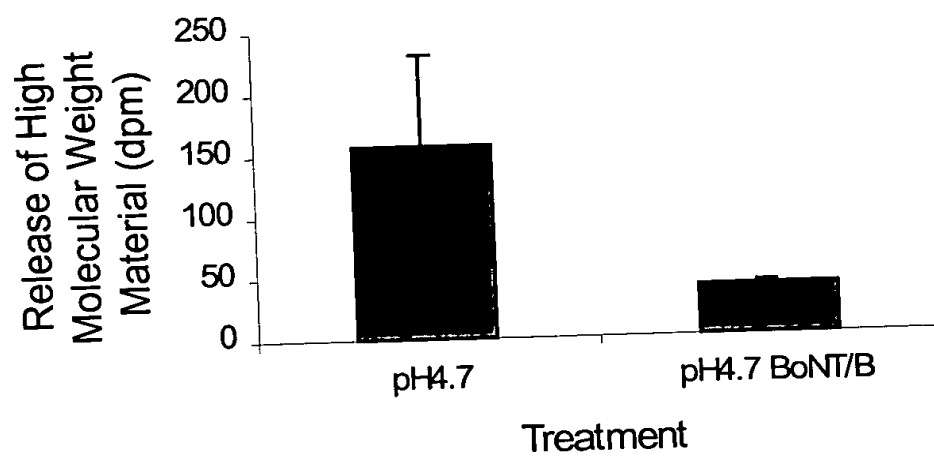


Fig. 8

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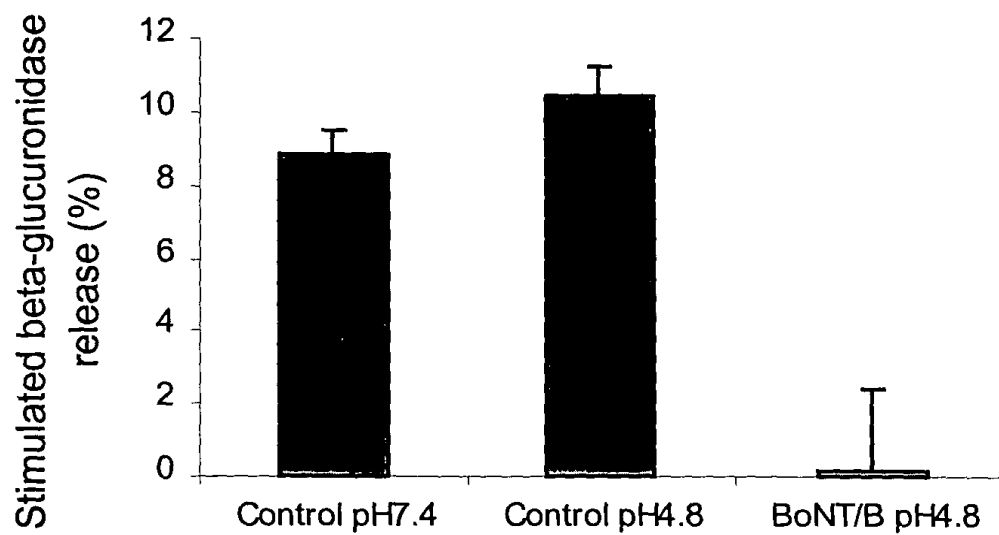




Fig. 9

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference <b>GWS/21755.01</b>		<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. <b>PCT/GB00/03681</b>	International filing date (day/month/year) <b>25/09/2000</b>	Priority date (day/month/year) <b>23/09/1999</b>	
International Patent Classification (IPC) or national classification and IPC <b>A61K47/48</b>			
Applicant <b>FOSTER, Keith Alan et al.</b>			
<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 10 sheets, including this cover sheet.</p> <p><input type="checkbox"/> This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of sheets.</p>			
<p>3. This report contains indications relating to the following items:</p> <ul style="list-style-type: none"><li>I <input checked="" type="checkbox"/> Basis of the report</li><li>II <input type="checkbox"/> Priority</li><li>III <input checked="" type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability</li><li>IV <input checked="" type="checkbox"/> Lack of unity of invention</li><li>V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</li><li>VI <input checked="" type="checkbox"/> Certain documents cited</li><li>VII <input type="checkbox"/> Certain defects in the international application</li><li>VIII <input type="checkbox"/> Certain observations on the international application</li></ul>			
Date of submission of the demand <b>02/04/2001</b>		Date of completion of this report <b>18.12.2001</b>	
Name and mailing address of the international preliminary examining authority:  <b>European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465</b>		Authorized officer  <b>Döpfer, K-P</b>  Telephone No. +49 89 2399 8547 	

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/03681

## 1. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

### Description, pages:

1-46 as originally filed

### Claims, No.:

1-39 as originally filed

### Drawings, sheets:

1/9-9/9 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/GB00/03681

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

**III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 1-3 (partially), 7-21 (complete), 22-30 (partially), 31-37(complete), 38-39 (partially).

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for the said claims Nos. 1-3 (partially), 7-21 (complete), 22-30 (partially), 31-37(complete), 38-39 (partially).

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

**IV. Lack of unity of invention**

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

**INTERNATIONAL PRELIMINARY  
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International application No. PCT/GB00/03681

- ☐ paid additional fees.
- ☐ paid additional fees under protest.
- ☐ neither restricted nor paid additional fees.
2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.
3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is
- ☐ complied with.
- ☒ not complied with for the following reasons:  
**see separate sheet**
4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:
- ☐ all parts.
- ☒ the parts relating to claims Nos. 1-3 (partially), 7-21 (complete), 22-30 (partially), 31-37(complete), 38-39 (partially).

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Yes: Claims
	No: Claims 1-6, 22-30, 38, 39
Inventive step (IS)	Yes: Claims
	No: Claims 1-6, 22-30, 38, 39
Industrial applicability (IA)	Yes: Claims 25-30 , 39
	No: Claims

2. Citations and explanations  
**see separate sheet**

**VI. Certain documents cited**

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/GB00/03681

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se separate sheet

**R Item I**

**Basis of the report**

**Re Item III**

**Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

1. No International Search Report has been drawn up for the subject-matter of present claims 1-3 (partially), 7-21 (complete), 22-30 (partially), 31-37 (complete), 38-39 (partially) (inventions 1.2-1.8 as listed under Item IV of this Opinion).  
According to Rule 66(1)(e) PCT no International Preliminary Examination is being carried out for the subject-matter of these claims.

**Re Item IV**

**Lack of unity of invention**

1. The International Preliminary Examination Authority agrees with the findings of the International Search Authority (see Form ISA 210 for the reasoning). Thus, the following separate (groups) of inventions are considered present in the application:

- 1.1 Claims: 1-3 (partially), 4-6 (complete), 22-30, 38, 39 (all partially)

Method of inhibiting secretion from an endocrine cell administering an agent comprising a first domain which cleaves proteins essential to exocytosis and a second domain that translocates into the cell, optionally further comprising a third domain with targeting activity namely ligands selected from those listed in claim 5.

- 1.2 Claims: 1-3 (partially), 7-9 (complete), 22-30, 38, 39 (all partially)

Method of inhibiting secretion from an inflammatory cell administering an agent comprising a first domain which cleaves proteins essential to exocytosis and a second domain that translocates into the cell, optionally further comprising a third domain with targeting activity namely ligands selected from those listed in claim 8.

**1.3 Claims: 1-3 (partially), 10-12 (complete), 22-30, 38, 39 (all partially)**

Method of inhibiting secretion from an endocrine cell administering an agent comprising a first domain which cleaves proteins essential to exocytosis and a second domain that translocates into the cell, optionally further comprising a third domain with targeting activity namely ligands selected from those listed in claim 11.

**1.4 Claims: 1-3 (partially), 13-15 (complete), 22-30, 38, 39 (all partially)**

Method of inhibiting secretion from an endocrine cell administering an agent comprising a first domain which cleaves proteins essential to exocytosis and a second domain that translocates into the cell, optionally further comprising a third domain with targeting activity namely ligands selected from those listed in claim 14.

**1.5 Claims: 1-3 (partially), 16-18 (complete), 22-30, 38, 39 (all partially)**

Method of inhibiting secretion from an endocrine cell administering an agent comprising a first domain which cleaves proteins essential to exocytosis and a second domain that translocates into the cell, optionally further comprising a third domain with targeting activity namely ligands selected from those listed in claim 17.

**1.6 Claims: 1-3 (partially), 19-21 (complete), 22-30, 38, 39 (all partially)**

Method of inhibiting secretion from an endocrine cell administering an agent comprising a first domain which cleaves proteins essential to exocytosis and a second domain that translocates into the cell, optionally further comprising a third domain with targeting activity namely ligands selected from those listed in claim 20.

**1.7 Claims: 37-39 (partially), 31, 32 (complete)**

Nucleic acid construct encoding a first domain which cleaves protein essential to exocytosis, a second domain that translocates into the cell and a third domain with

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/GB00/03681

targeting activity to non-neuronal cells. Agent for gene therapy and method of treatment using the same.

**1.8 Claims: 37 (partially), 33-36 (complete)**

Agent for gene therapy comprising a nucleic acid construct encoding a first domain which cleaves proteins essential to exocytosis, a second domain that translocates into the cell. Method of treatment using the same.

**Re Item V**

**Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1. Reference is made to the following documents:**

- D1: WO 01 14570 A (CHAN KUO CHION ;DOLLY J OLIVER (GB); LI YAN (GB); ALLERGAN SALES I) 1 March 2001 (2001-03-01)
- D2: WO 00 61192 A (ALLERGAN SALES INC) 19 October 2000 (2000-10-19)
- D3: WO 96 33273 A (DUGGAN MICHAEL JOHN ;FOSTER KEITH ALAN (GB); MICROBIOLOGICAL RES A) 24 October 1996 (1996-10-24)
- D4: WO 98 07864 A (FOSTER KEITH ALAN ;MICROBIOLOGICAL RES AUTHORITY (GB); QUINN CONRA) 26 February 1998 (1998-02-26)
- D5: WO 94 21300 A (FOSTER KEITH ALAN ;NORTH JOHN ROBERT (GB); HEALTH LAB SERVICE BOAR) 29 September 1994 (1994-09-29)
- D6: WO 00 10598 A (FOSTER KEITH ALAN ;MICROBIOLOGICAL RES AUTHORITY (GB); CHADDOK JO) 2 March 2000 (2000-03-02)
- D7: CHADDOK J A ET AL: 'INHIBITION OF VESICULAR SECRETION IN BOTH NEURONAL AND NONNEURONAL CELLS BY A RETARGETED ENDOPEPTIDASE DERIVATIVE OF CLOSTRIDIUM BOTULINUM NEUROTOXIN TYPE A' INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON, US, vol. 68, no. 5, May 2000 (2000-05), pages 2587-2593, XP001010268 ISSN: 0019-9567
- D8: NEMOZ-GAILLARD E ET AL: 'EXPRESSION OF SNARE PROTEINS IN ENTEROENDOCRINE CELL LINES AND FUNCTIONAL ROLE OF

TETANUS TOXIN-SENSITIVE PROTEINS IN CHOLECYSTOKININ  
RELEASE' FEBS LETTERS, ELSEVIER SCIENCE PUBLISHERS,  
AMSTERDAM, NL, vol. 425, no. 1, 20 March 1998 (1998-03-20), pages 66-  
70, XP001012518 ISSN: 0014-5793

2. Claims 1-6, 22-24, 38 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

3. Novelty and Inventive Step (Article 33(2)(3) PCT)

D4 (see the passages indicated in the ISR) discloses conjugates, methods and uses covered by the scope of present claims 1-6, 22-30, 38, 39.

D5 (see claims 15, 18, 20-24; examples 4, 6, 8-11) addresses agents consisting of three domains having characteristics as required by the subject-matter of the present application. Furthermore, methods and uses are disclosed which are considered pertinent for the novelty of present claims 1-6, 22-30, 38 and 39.

D7 is not regarded as prior art pursuant to Rule 64 PCT because the priority has been found validly claimed.

The combination of the teachings of D3 and D8 (D4 and D5 can also be taken as pertinent prior art for the assessment of inventive step by combining with the disclosure of either D3 or D8) would lead the skilled person also to the proposed solution of the posed problem, i.e. to provide conjugates which inhibit the exocytosis by e.g. protein processing in the target (non-neuronal) cell, i.e. in view of these documents inventive step is lacking for present claims 1-6, 22-30, 38 and 39.

**INTERNATIONAL PRELIMINARY  
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International application No. PCT/GB00/03681

4. Industrial applicability (Article 33(4) PCT)

The subject-matter of present claims 25-30 and 39 appear to comply with the requirements of industrial applicability as stipulated in Article 33(4) PCT.

**Re Item VI**

**Certain documents cited**

Certain published documents (Rule 70.10)

Application No Patent No	Publication date (day/month/year)	Filing date (day/month/year)	Priority date (valid claim) (day/month/year)
WO-A-01/14570	01.03.2001	25.08.2000	25.08.1999
WO-A-00/61192	19.10.2000	06.04.2000	08.04.1999
WO-A-00/10598	02.03.2000	25.08.1999	25.08.1998

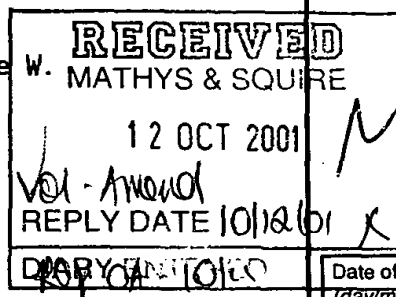
When entering the European phase, the disclosure of the above mentioned documents might be pertinent for the novelty of present claims 1-6, 22-30, 38, 39 under Art. 54(3) EPC.

PCT

From the INTERNATIONAL SEARCHING AUTHORITY

To:

MATHYS & SQUIRE  
Attn. SCHLICH, George W.  
100 Gray's Inn Road  
London WC1X 8AL  
UNITED KINGDOM



NOTIFICATION OF TRANSMITTAL OF  
THE INTERNATIONAL SEARCH REPORT  
OR THE DECLARATION

(PCT Rule 44.1)

Date of mailing (day/month/year)	10/10/2001
Applicant's or agent's file reference GWS/21755.01	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/GB 00/ 03681	International filing date (day/month/year) 25/09/2000
Applicant FOSTER, Keith Alan	

1. ☒ The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

**Filing of amendments and statement under Article 19:**

The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

**When?** The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

**Where?** Directly to the International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland  
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.

Within 19 months from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within 20 months from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority



European Patent Office, P.B. 5818 Patentlaan 2  
NL-2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Joannes Vergoosen

## NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

### INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

#### What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

#### When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

#### Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

#### How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

**The amendments must be made in the language in which the international application is to be published.**

#### What documents must/may accompany the amendments?

##### Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

**The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.**

## NOTES TO FORM PCT/ISA/220 (continued)

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

- (i) the claim is unchanged;
- (ii) the claim is cancelled;
- (iii) the claim is new;
- (iv) the claim replaces one or more claims as filed;
- (v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:  
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:  
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:  
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or  
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:  
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

### "Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

**It must be in the language in which the international application is to be published.**

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

### **Consequence if a demand for international preliminary examination has already been filed**

If, at the time of filing any amendments and any accompanying statement, under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the time of filing the amendments (and any statement) with the International Bureau, also file with the International Preliminary Examining Authority a copy of such amendments (and of any statement) and, where required, a translation of such amendments for the procedure before that Authority (see Rules 55.3(a) and 62.2, first sentence). For further information, see the Notes to the demand form (PCT/IPEA/401).

### **Consequence with regard to translation of the international application for entry into the national phase**

The applicant's attention is drawn to the fact that, upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

# PATENT COOPERATION TREATY

From the  
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

<p>To:</p> <p>MACLEAN, Martin Robert Mathys &amp; Squire 100 Gray's Inn Road London WC1X 8AL GRANDE BRETAGNE</p>	<div style="border: 1px solid black; padding: 5px; display: inline-block;"> <p><b>RECEIVED</b></p> <p><b>MATHYS &amp; SQUIRE</b></p> <p><b>24 DEC 2001</b></p> <p>REPLY DATE</p> <p>DIARY ENTERED</p> </div>
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## PCT

NOTIFICATION OF TRANSMITTAL OF  
THE INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT  
(PCT Rule 71.1)

Date of mailing (day/month/year) <span style="float: right;">18.12.2001</span>	
Applicant's or agent's file reference GWS/21755.01	<b>IMPORTANT NOTIFICATION</b>
International application No. PCT/GB00/03681	International filing date (day/month/year) 25/09/2000
Priority date (day/month/year) 23/09/1999	
Applicant FOSTER, Keith Alan et al.	

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.
4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/  <div style="display: flex; align-items: center;"> <div> <p>European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465</p> </div> </div>	Authorized officer:  <p>DA ROCHA, O.</p> <p>Tel. +49 89 2399-8101</p> <div style="text-align: right;"> </div>
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# PATENT COOPERATION TREATY

## PCT

### INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference <b>GWS/21755.01</b>	<b>FOR FURTHER ACTION</b>		See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. <b>PCT/GB00/03681</b>	International filing date (day/month/year) <b>25/09/2000</b>	Priority date (day/month/year) <b>23/09/1999</b>	
International Patent Classification (IPC) or national classification and IPC <b>A61K47/48</b>			
Applicant <b>FOSTER, Keith Alan et al.</b>			

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.



2. This REPORT consists of a total of 10 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☒ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☒ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand  <b>02/04/2001</b>	Date of completion of this report  <b>18.12.2001</b>
Name and mailing address of the international preliminary examining authority:   <b>European Patent Office</b> <b>D-80298 Munich</b> <b>Tel. +49 89 2399 - 0 Tx: 523656 epmu d</b> <b>Fax: +49 89 2399 - 4465</b>	Authorized officer:  <b>Döpfer, K-P</b>  Telephone No. <b>+49 89 2399 8547</b> <div style="text-align: right;">  </div>

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/03681

## I. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

### Description, pages:

1-46 as originally filed

### Claims, No.:

1-39 as originally filed

### Drawings, sheets:

1/9-9/9 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/GB00/03681

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

**III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 1-3 (partially), 7-21 (complete), 22-30 (partially), 31-37(complete), 38-39 (partially).

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for the said claims Nos. 1-3 (partially), 7-21 (complete), 22-30 (partially), 31-37(complete), 38-39 (partially).

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

**IV. Lack of unity of invention**

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/GB00/03681

- ☐ paid additional fees.
- ☐ paid additional fees under protest.
- ☐ neither restricted nor paid additional fees.
2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.
3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is
- ☐ complied with.
- ☒ not complied with for the following reasons:  
**see separate sheet**
4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:
- ☐ all parts.
- ☒ the parts relating to claims Nos. 1-3 (partially), 7-21 (complete), 22-30 (partially), 31-37 (complete), 38-39 (partially).

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1. Statement**

Novelty (N)	Yes:	Claims
	No:	Claims 1-6, 22-30, 38, 39
Inventive step (IS)	Yes:	Claims
	No:	Claims 1-6, 22-30, 38, 39
Industrial applicability (IA)	Yes:	Claims 25-30, 39
	No:	Claims

**2. Citations and explanations  
see separate sheet**

**VI. Certain documents cited**

**1. Certain published documents (Rule 70.10)**

and / or

**2. Non-written disclosures (Rule 70.9)**

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/GB00/03681

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see separate sheet

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/GB00/03681

**Re Item I**

**Basis of the report**

**Re Item III**

**Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

1. No International Search Report has been drawn up for the subject-matter of present claims 1-3 (partially), 7-21 (complete), 22-30 (partially), 31-37 (complete), 38-39 (partially) (inventions 1.2-1.8 as listed under Item IV of this Opinion). According to Rule 66(1)(e) PCT no International Preliminary Examination is being carried out for the subject-matter of these claims.

**Re Item IV**

**Lack of unity of invention**

1. The International Preliminary Examination Authority agrees with the findings of the International Search Authority (see Form ISA 210 for the reasoning). Thus, the following separate (groups) of inventions are considered present in the application:

- 1.1 Claims: 1-3 (partially), 4-6 (complete), 22-30, 38, 39 (all partially)

Method of inhibiting secretion from an endocrine cell administering an agent comprising a first domain which cleaves proteins essential to exocytosis and a second domain that translocates into the cell, optionally further comprising a third domain with targeting activity namely ligands selected from those listed in claim 5.

- 1.2 Claims: 1-3 (partially), 7-9 (complete), 22-30, 38, 39 (all partially)

Method of inhibiting secretion from an inflammatory cell administering an agent comprising a first domain which cleaves proteins essential to exocytosis and a second domain that translocates into the cell, optionally further comprising a third domain with targeting activity namely ligands selected from those listed in claim 8.

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/GB00/03681

**1.3 Claims: 1-3 (partially), 10-12 (complete), 22-30, 38, 39 (all partially)**

Method of inhibiting secretion from an endocrine cell administering an agent comprising a first domain which cleaves proteins essential to exocytosis and a second domain that translocates into the cell, optionally further comprising a third domain with targeting activity namely ligands selected from those listed in claim 11.

**1.4 Claims: 1-3 (partially), 13-15 (complete), 22-30, 38, 39 (all partially)**

Method of inhibiting secretion from an endocrine cell administering an agent comprising a first domain which cleaves proteins essential to exocytosis and a second domain that translocates into the cell, optionally further comprising a third domain with targeting activity namely ligands selected from those listed in claim 14.

**1.5 Claims: 1-3 (partially), 16-18 (complete), 22-30, 38, 39 (all partially)**

Method of inhibiting secretion from an endocrine cell administering an agent comprising a first domain which cleaves proteins essential to exocytosis and a second domain that translocates into the cell, optionally further comprising a third domain with targeting activity namely ligands selected from those listed in claim 17.

**1.6 Claims: 1-3 (partially), 19-21 (complete), 22-30, 38, 39 (all partially)**

Method of inhibiting secretion from an endocrine cell administering an agent comprising a first domain which cleaves proteins essential to exocytosis and a second domain that translocates into the cell, optionally further comprising a third domain with targeting activity namely ligands selected from those listed in claim 20.

**1.7 Claims: 37-39 (partially), 31, 32 (complete)**

Nucleic acid construct encoding a first domain which cleaves protein essential to exocytosis, a second domain that translocates into the cell and a third domain with

targeting activity to non-neuronal cells. Agent for gene therapy and method of treatment using the same.

**1.8 Claims: 37 (partially), 33-36 (complete)**

Agent for gene therapy comprising a nucleic acid construct encoding a first domain which cleaves proteins essential to exocytosis, a second domain that translocates into the cell. Method of treatment using the same.

**Re Item V**

**Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1. Reference is made to the following documents:**

- D1: WO 01 14570 A (CHAN KUO CHION ;DOLLY J OLIVER (GB); LI YAN (GB); ALLERGAN SALES I) 1 March 2001 (2001-03-01)
- D2: WO 00 61192 A (ALLERGAN SALES INC) 19 October 2000 (2000-10-19)
- D3: WO 96 33273 A (DUGGAN MICHAEL JOHN ;FOSTER KEITH ALAN (GB); MICROBIOLOGICAL RES A) 24 October 1996 (1996-10-24)
- D4: WO 98 07864 A (FOSTER KEITH ALAN ;MICROBIOLOGICAL RES AUTHORITY (GB); QUINN CONRA) 26 February 1998 (1998-02-26)
- D5: WO 94 21300 A (FOSTER KEITH ALAN ;NORTH JOHN ROBERT (GB); HEALTH LAB SERVICE BOAR) 29 September 1994 (1994-09-29)
- D6: WO 00 10598 A (FOSTER KEITH ALAN ;MICROBIOLOGICAL RES AUTHORITY (GB); CHADDOK JO) 2 March 2000 (2000-03-02)
- D7: CHADDOK J A ET AL: 'INHIBITION OF VESICULAR SECRETION IN BOTH NEURONAL AND NONNEURONAL CELLS BY A RETARGETED ENDOPEPTIDASE DERIVATIVE OF CLOSTRIDIUM BOTULINUM NEUROTOXIN TYPE A' INFECTION AND IMMUNITY,AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON,US, vol. 68, no. 5, May 2000 (2000-05), pages 2587-2593, XP001010268 ISSN: 0019-9567
- D8: NEMOZ-GAILLARD E ET AL: 'EXPRESSION OF SNARE PROTEINS IN ENTEROENDOCRINE CELL LINES AND FUNCTIONAL ROLE OF

TETANUS TOXIN-SENSITIVE PROTEINS IN CHOLECYSTOKININ  
RELEASE' FEBS LETTERS, ELSEVIER SCIENCE PUBLISHERS,  
AMSTERDAM, NL, vol. 425, no. 1, 20 March 1998 (1998-03-20), pages 66-  
70, XP001012518 ISSN: 0014-5793

2. Claims 1-6, 22-24, 38 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

3. Novelty and Inventive Step (Article 33(2)(3) PCT)

D4 (see the passages indicated in the ISR) discloses conjugates, methods and uses covered by the scope of present claims 1-6, 22-30, 38, 39.

D5 (see claims 15, 18, 20-24; examples 4, 6, 8-11) addresses agents consisting of three domains having characteristics as required by the subject-matter of the present application. Furthermore, methods and uses are disclosed which are considered pertinent for the novelty of present claims 1-6, 22-30, 38 and 39.

D7 is not regarded as prior art pursuant to Rule 64 PCT because the priority has been found validly claimed.

The combination of the teachings of D3 and D8 (D4 and D5 can also be taken as pertinent prior art for the assessment of inventive step by combining with the disclosure of either D3 or D8) would lead the skilled person also to the proposed solution of the posed problem, i.e. to provide conjugates which inhibit the exocytosis by e.g. protein processing in the target (non-neuronal) cell, i.e. in view of these documents inventive step is lacking for present claims 1-6, 22-30, 38 and 39.

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/GB00/03681

4. Industrial applicability (Article 33(4) PCT)

The subject-matter of present claims 25-30 and 39 appear to comply with the requirements of industrial applicability as stipulated in Article 33(4) PCT.

**Re Item VI**

**Certain documents cited**

Certain published documents (Rule 70.10)

Application No Patent No	Publication date (day/month/year)	Filing date (day/month/year)	Priority date (valid claim) (day/month/year)
WO-A-01/14570	01.03.2001	25.08.2000	25.08.1999
WO-A-00/61192	19.10.2000	06.04.2000	08.04.1999
WO-A-00/10598	02.03.2000	25.08.1999	25.08.1998

When entering the European phase, the disclosure of the above mentioned documents might be pertinent for the novelty of present claims 1-6, 22-30, 38, 39 under Art. 54(3) EPC.

## PATENT COOPERATION TREATY

PCT

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(PCT Article 20)

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